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(54) MULTIPLEX BRANCHED-CHAIN DNA ASSAYS

MULTIPLEX-ASSAYS FÜR VERZWEIGTKETTIGE DNA

ESSAIS RÉALISÉS SUR DE L'ADN À CHAÎNE RAMIFIÉE MULTIPLEXE

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Description

[0001] This application is a non-provisional utility patent application claiming priority to and benefit of the following prior provisional patent application: USSN 60/680,976, filed May 12, 2005, entitled "MULTIPLEX BRANCHED-CHAIN DNA ASSAYS" by Luo and Yang.

FIELD OF THE INVENTION

[0002] The present invention is in the field of nucleic acid detection. The invention includes methods for detecting the presence of 10 or more nucleic acids simultaneously in a single sample. The invention also includes compositions and kits related to the methods.

BACKGROUND OF THE INVENTION

[0003] Global gene expression profiling and other technologies have identified a large number of genes whose expression is altered, e.g., in diseased tissues or in tissues and cells treated with pharmaceutical agents (Lockhart and Winzeler (2000) "Genomics, gene expression and DNA arrays" Nature 405:827-36 and Gunther et al. (2003) "Prediction of clinical drug efficacy by classification of drug-induced genomic expression profiles in vitro" Proc Natl Acad Sci USA 100:9608-13). Such genes are being increasingly used as biomarkers in disease diagnosis, staging, and prognosis (Golub et al. (1999) "Molecular classification of cancer: class discovery and class prediction by gene expression monitoring" Science 286:531-7); target identification, validation and pathway analysis (Roberts et al. (2000) "Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles" Science 287:873-80); drug screening (Hamadeh et al. (2002) "Prediction of compound signature using high density gene expression profiling" Toxicol Sci 67:232-40); and studies of drug efficacy, structure-activity relationship, toxicity, and drug-target interactions (Gerhold et al. (2001) "Monitoring expression of genes involved in drug metabolism and toxicology using DNA microarrays" Physiol Genomics 5:161-70 and Thomas et al. (2001) "Identification of toxicologically predictive gene sets using cDNA microarrays" Mol Pharmacol 60:1189-94). As biomarkers are identified, their involvement in disease management and drug development will need to be evaluated in higher throughput and broader populations of samples. Simpler and more flexible expression profiling technology that allows the expression analysis of multiple genes with higher data quality and higher throughput is therefore needed.

[0004] Levels of RNA expression have traditionally been measured using Northern blot and nuclease protection assays. However, these approaches are time-consuming and have limited sensitivity, and the data generated are more qualitative than quantitative in nature. Greater sensitivity and quantification is possible with reverse transcription polymerase chain reaction (RT-PCR) based methods, such as quantitative real-time RT-PCR, but these approaches have low multiplex capabilities (Bustin (2002) "Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems" J Mol Endocrinol 29:23-39 and Bustin and Nolan (2004) "Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction" J Biomol Tech. 15:155-66). Microarray technology has been widely used in discovery research, but its moderate sensitivity and its relatively long experimental procedure have limited its use in high throughput expression profiling applications (Epstein and Butow (2000) "Microarray technology - enhanced versatility, persistent challenge" Curr Opin Biotechnol. 11:36-41).

[0005] Most of the current methods of mRNA quantification require RNA isolation, reverse transcription, and target amplification, each of which introduces variability that leads to low overall assay precision. Recently, a multiplex screening assay for mRNA quantification combining nuclease protection with luminescent array detection was reported (Martel et al. (2002) "Multiplexed screening assay for mRNA combining nuclease protection with luminescent array detection" Assay Drug Dev Technol. 1:61-71). Although this assay has the advantage of measuring mRNA transcripts directly from cell lysates, limited assay sensitivity and reproducibility were reported. Another multiplex mRNA assay without the need for RNA isolation was also reported (Tian et al. (2004) "Multiplex mRNA assay using electrophoretic tags for high-throughput gene expression analysis" Nucleic Acids Res. 32:e126). This assay couples the primary Invader® mRNA assay with small fluorescent molecule eTags that can be distinguished by capillary electrophoresis through distinct charge-to-mass ratios of eTags. However, this assay requires the use of a specially designed and synthesized set of eTagged signal probes, complicated capillary electrophoresis equipment, and a special data analysis package.

[0006] Among other aspects, the present invention provides methods that overcome the above noted limitations and permit rapid, simple, and sensitive detection of multiple mRNAs (and/or other nucleic acids) simultaneously. A complete understanding of the invention will be obtained upon review of the following.

SUMMARY OF THE INVENTION

[0007] In one aspect, the present invention provides methods of detecting 10 or more nucleic acids of interest in a multiplex branched-chain DNA assay. Different nucleic acids are captured through cooperative hybridization events on different, identifiable subsets of particles or at different selected positions on a spatially addressable solid support. Compositions and kits related to the methods are also provided.

[0008] A first general class of embodiments provides methods of detecting 10 or more nucleic acids of interest. In the methods, a sample, a pooled population of particles, and 10 or more subsets of n capture extenders, wherein n is at least two, are provided. The sample comprises or is suspected of comprising the nucleic acids of interest. The pooled population of particles includes 10 or more subsets of particles, and a plurality of the particles in each subset are distinguishable from a plurality of the particles in every other subset. The particles in each subset have associated therewith a different capture probe. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected subset of the particles. Each nucleic acid of interest can thus, by hybridizing to its corresponding subset of n capture extenders which are in turn hybridized to a corresponding capture probe, be associated with an identifiable subset of the particles.

[0009] The sample, the pooled population of particles, and the subsets of n capture extenders are contacted, any nucleic acid of interest present in the sample is hybridized to its corresponding subset of n capture extenders, and the subset of n capture extenders is hybridized to its corresponding capture probe. The hybridizing the nucleic acid of interest to the n capture extenders and the n capture extenders to the corresponding capture probe captures the nucleic acid on the subset of particles with which the capture extenders are associated. The hybridizing the subset of n capture extenders to the corresponding capture probe is performed at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and its corresponding capture probe.

[0010] To determine which subsets of particles have a nucleic acid of interest captured on the particles, one or more label extenders and a label probe system comprising a label are hybridized to any nucleic acid of interest captured on the particles, and at least a portion of the particles from each subset are identified and the presence or absence of the label is detected on those particles. Since a correlation exists between a particular subset of particles and a particular nucleic acid of interest, which subsets of particles have the label present indicates which of the nucleic acids of interest were present in the sample.

[0011] The methods are useful for multiplex detection of nucleic acids, optionally highly multiplex detection. Thus, the 10 or more nucleic acids of interest (i.e., the nucleic acids to be detected) optionally comprise 20 or more, 30 or more, 40 or more, 50 or more, or even 100 or more nucleic acids of interest. A like number of subsets of particles and subsets of capture extenders are provided; thus, the or more subsets of particles can comprise 20 or more, 30 or more, 40 or more, 50 or more, or even 100 or more subsets of particles, while the 10 or more subsets of n capture extenders can comprise 20 or more, 30 or more, 40 or more, 50 or more, or even 100 or more subsets of n capture extenders.

[0012] In one class of embodiments, the particles are microspheres. The microspheres of each subset can be distinguishable from those of the other subsets, e.g., on the basis of their fluorescent emission spectrum, their diameter, or a combination thereof.

[0013] As noted, each of the 10 or more subsets of capture extenders includes n capture extenders, where n is at least two. Preferably, n is at least three, and n can be at least four or at least five or more. Typically, but not necessarily, n is at most ten. The n capture extenders in a subset hybridize to nonoverlapping polynucleotide sequences in the corresponding nucleic acid of interest. The nonoverlapping polynucleotide sequences can, but need not be, consecutive within the nucleic acid of interest.

[0014] Each capture extender is capable of hybridizing to its corresponding capture probe. The capture extender typically includes a polynucleotide sequence C-1 that is complementary to a polynucleotide sequence C-2 in its corresponding capture-probe. In one aspect, C-1 and C-2 are 20 nucleotides or less in length. In one class of embodiments, C-1 and C-2 are between 9 and 17 nucleotides in length (inclusive), preferably between 12 and 1S nucleotides (inclusive).

[0015] As noted, the hybridizing the subset of n capture extenders to the corresponding capture probe is performed at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and its corresponding capture probe. The hybridization temperature is typically about 5˚C or more greater than the T_m, e.g., about 7˚C or more, about 10˚C or more, about 12˚C or more, about 15˚C or more, about 17˚C or more, or even about 20˚C or more greater than the T_m.

[0016] In one class of embodiments, contacting the sample, the pooled population of particles, and the subsets of n capture extenders comprises combining the sample with the subsets of n capture extenders to form a mixture, and then combining the mixture with the pooled population of particles.

[0017] In a preferred class of embodiments, hybridizing one or more label extenders and a label probe system to any nucleic acid of interest captured on the particles comprises providing 10 or more subsets of one or more label extenders, wherein each subset of label extenders is capable of hybridizing to one of the nucleic acids of interest, hybridizing any nucleic acid of interest captured on the particles to its corresponding subset of label extenders, and hybridizing the label probe system to the label extenders. The 10 or more subsets of label extenders can include, e.g., 20 or more, 30 or more, 40 or more, 50 or more, or even 100 or more subsets of label extenders. The hybridizations can be performed simultaneously or sequentially, in essentially any order. In one embodiment, any nucleic acid of interest present in the sample is hybridized to its corresponding subset of label extenders and to its corresponding subset of n capture extenders, then the subset of n capture extenders is hybridized to its corresponding capture probe.

[0018] The label probe system optionally includes an amplification multimer and a plurality of label probes, wherein the amplification multimer is capable of hybridizing to a label extender and to a plurality of label probes. In one class of embodiments, the label probe comprises the label. In one aspect, the label is a fluorescent label, and detecting the presence of the label on the particles comprises detecting a fluorescent signal from the label.

[0019] The methods can optionally be used to quantitate the amounts of the nucleic acids of interest present in the sample. For example, in one class of embodiments, an intensity of a signal from the label is measured, e.g., for each subset of particles, and correlated with a quantity of the corresponding nucleic acid of interest present.

[0020] At any of various steps, materials not captured on the particles are optionally separated from the particles. For example, after the capture extenders, nucleic acids, label extenders, blocking probes, and particle-bound capture probes are hybridized, the particles are optionally washed to remove unbound nucleic acids and probes; after the label extenders and amplification multimer are hybridized, the particles are optionally washed to remove unbound amplification multimer; and/or after the label probes are hybridized to the amplification multimer, the particles are optionally washed to remove unbound label probe prior to detection of the label.

[0021] The methods can be used to detect the presence of the nucleic acids of interest in essentially any type of sample. For example, the sample can be derived from an animal, a human, a plant, a cultured cell, a virus, a bacterium, a pathogen, and/or a microorganism. The sample optionally includes a cell lysate, an intercellular fluid, a bodily fluid (including, but not limited to, blood, serum, saliva, urine, sputum, or spinal fluid), and/or a conditioned culture medium, and is optionally derived from a tissue (e.g., a tissue homogenate), a biopsy, and/or a tumor. Similarly, the nucleic acids can be essentially any desired nucleic acids. As just a few examples, the nucleic acids of interest can be derived from one or more of an animal, a human, a plant, a cultured cell, a microorganism, a virus, a bacterium, or a pathogen. In one class of embodiments, the 10 or more nucleic acids of interest comprise 10 or more mRNAs.

[0022] In one class of embodiments, at least one of the nucleic acids of interest is present in the sample in a non-zero amount of 200 amol or less, 150 amol or less, 100 amol or less, 50 amol or less, 10 amol or less, 1 amol or less, or even 0.1 amol or less. In one exemplary class of embodiments, the sample includes a first nucleic acid of interest, and at least 30%, at least 50%, at least 80%, at least 90%, at least 95%, or even at least 99% of a total amount of the first nucleic acid present in the sample is captured on a first subset of particles. Second, third, etc. nucleic acids can similarly be quantitatively captured. Such quantitative capture can occur without capture of a significant amount of undesired nucleic acids, even those of very similar sequence to the nucleic acid of interest.

[0023] Thus, in one class of embodiments, the sample comprises or is suspected of comprising a first nucleic acid of interest and a second nucleic acid which has a polynucleotide sequence which is 95% or more identical to that of the first nucleic acid (e.g., 96% or more, 97% or more, 98% or more, or even 99% or more identical). The first nucleic acid, if present in the sample, is captured on a first subset of particles, while the . second nucleic acid comprises 1% or less of a total amount of nucleic acid captured on the first subset of particles (e.g., 0.5% or less, 0.2% or less, or even 0.1% or less).

[0024] As just one example of how closely related nucleic acids can be distinguished using the methods of the invention, different splice variants of a given mRNA can be distinguished. Thus, in one class of embodiments, the sample comprises a first nucleic acid of interest and a second nucleic acid, where the first nucleic acid is a first splice variant and the second nucleic acid is a second splice variant of the given mRNA. A first subset of n capture extenders is capable of hybridizing to the first splice variant, of which at most n-1 capture extenders are capable of hybridizing to the second splice variant. Preferably, hybridization of the n capture extenders to the first splice variant captures the first splice variant on a first subset of particles while hybridization of the at most n-1 capture extenders to the second splice variant does not capture the second splice variant on the first subset of particles.

[0025] Another general class of embodiments provides a composition for detecting 10 or more nucleic acids of interest that includes 10 or more subsets of particles, 10 or more subsets of n capture extenders, wherein n is at least two, 10 or more subsets of one or more label extenders, and a label probe system comprising a label. A plurality of the particles in each subset are distinguishable from a plurality of the particles in every other subset. The particles in each subset have associated therewith a different capture probe. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected subset of the particles. When the nucleic acid of interest corresponding to a subset of n capture extenders is present in the composition and is hybridized to the subset of n capture extenders, which are hybridized to the corresponding capture probe, the nucleic acid of interest is hybridized to the subset of n capture extenders at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and the capture probe.

[0026] Essentially all of the features noted for the embodiments above apply to these embodiments as well, as relevant; for example, with respect to number of capture extenders per subset, composition of the label probe system, type of label, inclusion of blocking probes, configuration of the capture extenders, capture probes, label extenders, and/or blocking probes, number of nucleic acids of interest and of subsets of particles, capture extenders and label extenders, source of the sample and/or nucleic acids, and/or the like.

[0027] A related general class of embodiments provides a composition comprising 10 or more subsets of particles, 10 or more subsets of n capture extenders, wherein n is at least two, 10 or more subsets of one or more label extenders, a label probe system comprising a label, and at least a first nucleic acid of interest. A plurality of the particles in each subset are distinguishable from a plurality of the particles in every other subset. The particles in each subset have associated therewith a different capture probe. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected subset of the particles. In this class of embodiments, the composition is maintained at a hybridization temperature, which hybridization temperature is greater than a melting temperature T_m of a complex between each individual capture extender and its corresponding capture probe. The first nucleic acid of interest is hybridized to a first subset of n first capture extenders, which first capture extenders are hybridized to a first capture probe.

[0028] Essentially all of the features noted for the embodiments above apply to these embodiments as well, as relevant; for example, with respect to number of capture extenders per subset, composition of the label probe system, type of label, inclusion of blocking probes, configuration of the capture extenders, capture probes, label extenders, and/or blocking probes, number of nucleic acids of interest and of subsets of particles, capture extenders and label extenders, source of the sample and/or nucleic acids, and/or the like.

[0029] Discussed herein is a kit for detecting two or more nucleic acids of interest. The kit includes two or more subsets of particles, two or more subsets of n capture extenders, wherein n is at least two, two or more subsets of one or more label extenders, and a label probe system comprising a label, packaged in one or more containers. A plurality of the particles in each subset are distinguishable from a plurality of the particles in every other subset. The particles in each subset have associated therewith a different capture probe. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected subset of the particles. When the nucleic acid of interest corresponding to a subset of n capture extenders is hybridized to the subset of n capture extenders, which are hybridized to the corresponding capture probe, the nucleic acid of interest is hybridized to the subset of n capture extenders at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and the capture probe. The kit optionally also includes instructions for using the kit to capture and detect the nucleic acids of interest, one or more buffered solutions (e.g., lysis buffer, diluent, hybridization buffer, and/or wash buffer), standards comprising one or more nucleic acids at known concentration, and/or the like.

[0030] Essentially all of the features noted for the embodiments above apply to the kits as well, as relevant; for example, with respect to number of capture extenders per subset, composition of the label probe system, type of label, inclusion of blocking probes, configuration of the capture extenders, capture probes, label extenders, and/or blocking probes, number of nucleic acids of interest and of subsets of particles, capture extenders and label extenders, source of the sample and/or nucleic acids, and/or the like.

[0031] Yet another general class of embodiments includes methods of detecting 10 or more nucleic acids of interest. In the methods, a sample, a solid support, and 10 or more subsets of n capture extenders, wherein n is at least two, are provided. The sample comprises or is suspected of comprising the nucleic acids of interest. The solid support comprises 10 or more capture probes, each of which is provided at a selected position on the solid support. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected position on the solid support. Each nucleic acid of interest can thus, by hybridizing to its corresponding subset of n capture extenders which are in turn hybridized to a corresponding capture probe, be associated with, e.g., a known, predetermined location on the solid support. The sample, the solid support, and the subsets of n capture extenders are contacted, any nucleic acid of interest present in the sample is hybridized to its corresponding subset of n capture extenders, and the subset of n capture extenders is hybridized to its corresponding capture probe. The hybridizing the nucleic acid of interest to the n capture extenders and the n capture extenders to the corresponding capture probe captures the nucleic acid on the solid support at the selected position with which the capture extenders are associated. To determine which positions on the solid support have a nucleic acid of interest captured at that position, one or more label extenders and a label probe system comprising a label are hybridized to any nucleic acid of interest captured on the solid support, and the presence or absence of the label at the selected positions on the solid support is detected. Since a correlation exists between a particular position on the support and a particular nucleic acid of interest, which positions have a label present indicates which of the nucleic acids of interest were present in the sample.

[0032] Hybridizing the subset of n capture extenders to the corresponding capture probe is optionally performed at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and its corresponding capture probe.

[0033] Essentially all of the features noted for the embodiments above apply to these embodiments as well, as relevant; for example, with respect to number of capture extenders per subset, composition of the label probe system, type of label, inclusion of blocking probes, configuration of the capture extenders, capture probes, label extenders, and/or blocking probes, number of nucleic acids of interest and of selected positions on the solid support, capture extenders and label extenders, source of the sample and/or nucleic acids, and/or the like.

[0034] Another general class of embodiments provides a composition for detecting 10 or more nucleic acids of interest, the composition that includes a solid support comprising 10 or more capture probes, each of which is provided at a selected position on the solid support, 10 or more subsets of n capture extenders, wherein n is at least two, or more subsets of one or more label extenders, and a label probe system comprising a label. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected position on the solid support.

[0035] Essentially all of the features noted for the embodiments above apply to these embodiments as well, as relevant; for example, with respect to number of capture extenders per subset, composition of the label probe system, type of label, inclusion of blocking probes, configuration of the capture extenders, capture probes, label extenders, and/or blocking probes, number of nucleic acids of interest and of selected positions on the solid support, capture extenders and label extenders, source of the sample and/or nucleic acids, and/or the like.

[0036] Discussed herein is a kit for detecting two or more nucleic acids of interest. The kit includes a solid support comprising two or more capture probes, each of which is provided at a selected position on the solid support, two or more subsets of n capture extenders, wherein n is at least two, two or more subsets of one or more label extenders, and a label probe system comprising a label, packaged in one or more containers. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected position on the solid support. The kit optionally also includes instructions for using the kit to capture and detect the nucleic acids of interest, one or more buffered solutions (e.g., lysis buffer, diluent, hybridization buffer, and/or wash buffer), standards comprising one or more nucleic acids at known concentration, and/or the like.

[0037] Essentially all of the features noted for the embodiments above apply to these kits as well, as relevant; for example, with respect to number of capture extenders per subset, composition of the label probe system, type of label, inclusion of blocking probes, configuration of the capture extenders, capture probes, label extenders, and/or blocking probes, number of nucleic acids of interest and of selected positions on the solid support, capture extenders and label extenders, source of the sample and/or nucleic acids, and/or the like.

BRIEF DESCRIPTION OF THE DRAWINGS

[0038] **Figure 1** schematically illustrates a typical standard bDNA assay.

[0039] **Figure 2 Panels A-E** schematically depict a multiplex bDNA assay, in which the nucleic acids of interest are captured on distinguishable subsets of microspheres and then detected.

[0040] **Figure 3** schematically depicts an exemplary embodiment in which two splice variants are specifically captured on distinguishable subsets of microspheres and then detected.

[0041] **Figure 4 Panels A-D** schematically depict a multiplex bDNA assay, in which the nucleic acids of interest are captured at selected positions on a solid support and then detected. **Panel A** shows a top view of the solid support, while **Panels B-D** show the support in cross-section.

[0042] **Figure 5 Panels A-C** schematically depict an overview of a multiplex bDNA assay.

[0043] **Figure 6 Panel A** schematically depicts simple hybridization of a single CE to a CP and a target mRNA. **Panel B** schematically depicts cooperative hybridization of CEs to a CP and a target mRNA. **Panel C** depicts a bar graph illustrating simple hybridization between a CE and CP under standard bDNA assay conditions. **Panel D** depicts a line graph illustrating cooperative hybridization between CEs and CPs under standard bDNA assay conditions.

[0044] **Figure 7 Panel A** depicts a graph illustrating evaluation of the cytokine panel for cross-reactivity. Individual mRNA transcript names are presented on the x-axis, bead subsets are identified by number on the y-axis, and fluorescent signal is graphed on the z-axis. **Panel B** depicts a line graph of fluorescent intensity observed for a dilution series of dapB transcript in the presence (squares) or absence (diamonds) of 0.2 μg total RNA, for evaluation of specificity. **Panel C** depicts a line graph of fluorescent intensity observed for a dilution series of IL10 transcript in the presence (squares) or absence (diamonds) of 0.2 μg total RNA, for evaluation of specificity. **Panel D** depicts a line graph of fluorescent intensity for a dilution series of all the transcripts in the cytokine panel, for evaluation of assay sensitivity and dynamic range.

[0045] **Figure 8 Panel A** depicts a bar graph illustrating induction of cytokine gene expression upon PMA/LPS treatment of U937 cells, as detected by a 10-plex bDNA assay. **Panel B** depicts a bar graph illustrating induction of cytokine gene expression upon PMA/LPS treatment of U937 cells, as detected by single-plex bDNA assays using the same cell lysates.

[0046] **Figure 9 Panel A** depicts bar graphs illustrating time-dependent expression of pro- and anti-apoptotic genes upon TNFα treatment of HeLa cells, as detected by a 10-plex bDNA assay. **Panel B** depicts bar graphs illustrating expression of pro- and anti-apoptotic genes in untreated cells and in TNFα-treated cells, 3 hours after treatment, as detected by single-plex bDNA assays (top) and a 10-plex bDNA assay (bottom).

[0047] Schematic figures are not necessarily to scale.

DEFINITIONS

[0048] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. The following definitions supplement those in the art and are directed to the current application and are not to be imputed to any related or unrelated case, e.g., to any commonly owned patent or application. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. Accordingly, the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting.

[0049] As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a molecule" includes a plurality of such molecules, and the like.

[0050] The term "about" as used herein indicates the value of a given quantity varies by +/-10% of the value, or optionally +/- 5% of the value, or in some embodiments, by +/-1 % of the value so described.

[0051] The term "polynucleotide" (and the equivalent term "nucleic acid") encompasses any physical string of monomer units that can be corresponded to a string of nucleotides, including a polymer of nucleotides (e.g., a typical DNA or RNA polymer), peptide nucleic acids (PNAs), modified oligonucleotides (e.g., oligonucleotides comprising nucleotides that are not typical to biological RNA or DNA, such as 2'-O-methylated oligonucleotides), and the like. The nucleotides of the polynucleotide can be deoxyribonucleotides, ribonucleotides or nucleotide analogs, can be natural or non-natural, and can be unsubstituted, unmodified, substituted or modified. The nucleotides can be linked by phosphodiester bonds, or by phosphorothioate linkages, methylphosphonate linkages, boranophosphate linkages, or the like. The polynucleotide can additionally comprise non-nucleotide elements such as labels, quenchers, blocking groups, or the like. The polynucleotide can be, e.g., single-stranded or double-stranded.

[0052] A "polynucleotide sequence" or "nucleotide sequence" is a polymer of nucleotides (an oligonucleotide, a DNA, a nucleic acid, etc.) or a character string representing a nucleotide polymer, depending on context. From any specified polynucleotide sequence, either the given nucleic acid or the complementary polynucleotide sequence (e.g., the complementary nucleic acid) can be determined.

[0053] Two polynucleotides "hybridize" when they associate to form a stable duplex, e.g., under relevant assay conditions. Nucleic acids hybridize due to a variety of well characterized physico-chemical forces, such as hydrogen bonding, solvent exclusion, base stacking and the like. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes, part I chapter 2, "Overview of principles of hybridization and the strategy of nucleic acid probe assays" (Elsevier, New York), as well as in Ausubel, *infra.*

[0054] The "T_m" (melting temperature) of a nucleic acid duplex under specified conditions (e.g., relevant assay conditions) is the temperature at which half of the base pairs in a population of the duplex are disassociated and half are associated. The T_m for a particular duplex can be calculated and/or measured, e.g., by obtaining a thermal denaturation curve for the duplex (where the T_m is the temperature corresponding to the midpoint in the observed transition from double-stranded to single-stranded form).

[0055] The term "complementary" refers to a polynucleotide that forms a stable duplex with its "complement," e.g., under relevant assay conditions. Typically, two polynucleotide sequences that are complementary to each other have mismatches at less than about 20% of the bases, at less than about 10% of the bases, preferably at less than about 5% of the bases, and more preferably have no mismatches.

[0056] A "capture extender" or "CE" is a polynucleotide that is capable of hybridizing to a nucleic acid of interest and to a capture probe. The capture extender typically has a first polynucleotide sequence C-1, which is complementary to the capture probe, and a second polynucleotide sequence C-3, which is complementary to a polynucleotide sequence of the nucleic acid of interest. Sequences C-1 and C-3 are typically not complementary to each other. The capture extender is preferably single-stranded.

[0057] A "capture probe" or "CP" is a polynucleotide that is capable of hybridizing to at least one capture extender and that is tightly bound (e.g., covalently or noncovalently, directly or through a linker, e.g., streptavidin-biotin or the like) to a solid support, a spatially addressable solid support, a slide, a particle, a microsphere, or the like. The capture probe typically comprises at least one polynucleotide sequence C-2 that is complementary to polynucleotide sequence

C-1 of at least one capture extender. The capture probe is preferably single-stranded.

[0058] A "label extender" or "LE" is a polynucleotide that is capable of hybridizing to a nucleic acid of interest and to a label probe system. The label extender typically has a first polynucleotide sequence L-1, which is complementary to a polynucleotide sequence of the nucleic acid of interest, and a second polynucleotide sequence L-2, which is complementary to a polynucleotide sequence of the label probe system (e.g., L-2 can be complementary to a polynucleotide sequence of an amplification multimer, a preamplifier, a label probe, or the like). The label extender is preferably single-stranded.

[0059] A "label" is a moiety that facilitates detection of a molecule. Common labels in the context of the present invention include fluorescent, luminescent, light-scattering, and/or colorimetric labels. Suitable labels include enzymes and fluorescent moieties, as well as radionuclides, substrates, cofactors, inhibitors, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Many labels are commercially available and can be used in the context of the invention.

[0060] A "label probe system" comprises one or more polynucleotides that collectively comprise a label and a polynucleotide sequence M-1, which is capable of hybridizing to at least one label extender. The label provides a signal, directly or indirectly. Polynucleotide sequence M-1 is typically complementary to sequence L-2 in the label extenders. The label probe system can include a plurality of label probes (e.g., a plurality of identical label probes) and an amplification multimer; it optionally also includes a preamplifier or the like, or optionally includes only label probes, for example.

[0061] An "amplification multimer" is a polynucleotide comprising a plurality of polynucleotide sequences M-2, typically (but not necessarily) identical polynucleotide sequences M-2. Polynucleotide sequence M-2 is complementary to a polynucleotide sequence in the label probe. The amplification multimer also includes at least one polynucleotide sequence that is capable of hybridizing to a label extender or to a nucleic acid that hybridizes to the label extender, e.g., a preamplifier. For example, the amplification multimer optionally includes at least one polynucleotide sequence M-1; polynucleotide sequence M-1 is typically complementary to polynucleotide sequence L-2 of the label extenders. Similarly, the amplification multimer optionally includes at least one polynucleotide sequence that is complementary to a polynucleotide sequence in a preamplifier. The amplification multimer can be, e.g., a linear or a branched nucleic acid. As noted for all polynucleotides, the amplification multimer can include modified nucleotides and/or nonstandard internucleotide linkages as well as standard deoxyribonucleotides, ribonucleotides, and/or phosphodiester bonds. Suitable amplification multimers are described, for example, in USPN 5,635,352, USPN 5,124,246, USPN 5,710,264, and USPN 5,849,481.

[0062] A "label probe" or "LP" is a single-stranded polynucleotide that comprises a label (or optionally that is configured to bind to a label) that directly or indirectly provides a detectable signal. The label probe typically comprises a polynucleotide sequence that is complementary to the repeating polynucleotide sequence M-2 of the amplification multimer; however, if no amplification multimer is used in the bDNA assay, the label probe can, e.g., hybridize directly to a label extender.

[0063] A "preamplifier" is a nucleic acid that serves as an intermediate between at least one label extender and amplification multimer. Typically, the preamplifier is capable of hybridizing simultaneously to at least one label extender and to a plurality of amplification multimers.

[0064] A "microsphere" is a small spherical, or roughly spherical, particle. A microsphere typically has a diameter less than about 1000 micrometers (e.g., less than about 100 micrometers, optionally less than about 10 micrometers).

[0065] A "microorganism" is an organism of microscopic or submicroscopic size. Examples include, but are not limited to, bacteria, fungi, yeast, protozoans, microscopic algae (e.g., unicellular algae), viruses (which are typically included in this category although they are incapable of growth and reproduction outside of host cells), subviral agents, viroids, and mycoplasma.

[0066] A variety of additional terms are defined or otherwise characterized herein.

DETAILED DESCRIPTION

[0067] The present invention provides methods and compositions for multiplex detection of nucleic acids. Nucleic acids of interest are captured to distinct subsets of particles or to selected positions on a solid support through cooperative hybridization, and they are then detected in a branched-chain DNA assay.

[0068] Branched-chain DNA (bDNA) signal amplification technology has been used, e.g., to detect and quantify mRNA transcripts in cell lines and to determine viral loads in blood. The bDNA assay is a sandwich nucleic acid hybridization procedure that enables direct measurement of mRNA expression, e.g., from crude cell lysate. It provides direct quantification of nucleic acid molecules at physiological levels. Several advantages of the technology distinguish it from other DNA/RNA amplification technologies, including linear amplification, good sensitivity and dynamic range, great precision and accuracy, simple sample preparation procedure, and reduced sample-to-sample variation.

[0069] In brief, in a typical bDNA assay for gene expression analysis (**Figure 1**), a target mRNA whose expression is to be detected is released from cells and captured by a Capture Probe (CP) on a solid surface (e.g., a well of a microtiter plate) through synthetic oligonucleotide probes called Capture Extenders (CEs). Each capture extender has a first polynucleotide sequence that can hybridize to the target mRNA and a second polynucleotide sequence that can hybridize to the capture probe. Typically, two or more capture extenders are used. Probes of another type, called Label Extenders (LEs), hybridize to different sequences on the target mRNA and to sequences on an amplification multimer. Additionally, Blocking Probes (BPs) are often used to reduce non-specific target probe binding. A probe set for a given mRNA thus consists of CEs, LEs, and optionally BPs for the target mRNA. The CEs, LEs, and BPs are complementary to nonoverlapping sequences in the target mRNA, and are typically, but not necessarily, contiguous.

[0070] Signal amplification begins with the binding of the LEs to the target mRNA. An amplification multimer is then typically hybridized to the LEs. The amplification multimer has multiple copies of a sequence that is complementary to a label probe (it is worth noting that the amplification multimer is typically, but not necessarily, a branched-chain nucleic acid; for example, the amplification multimer can be a branched, forked, or comb-like nucleic acid or a linear nucleic acid). A label, for example, alkaline phosphatase, is covalently attached to each label probe. (Alternatively, the label can be noncovalently bound to the label probes.) In the final step, labeled complexes are detected, e.g., by the alkaline phosphatase-mediated degradation of a chemilumigenic substrate, e.g., dioxetane. Luminescence is reported as relative light unit (RLUs) on a microplate reader. The amount of chemiluminescence is proportional to the level of mRNA expressed from the target gene.

[0071] In the preceding example, the amplification multimer and the label probes comprise a label probe system. In another example, the label probe system also comprises a preamplifier, e.g., as described in USPN 5,635,352 and USPN 5,681,697, which further amplifies the signal from a single target mRNA. In yet another example, the label extenders hybridize directly to the label probes and no amplification multimer or preamplifier is used, so the signal from a single target mRNA molecule is only amplified by the number of distinct label extenders that hybridize to that mRNA.

[0072] Basic bDNA assays have been well described. See, e.g., USPN 4,868,105 to Urdea et al. entitled "Solution phase nucleic acid sandwich assay"; USPN 5,635,352 to Urdea et al. entitled "Solution phase nucleic acid sandwich assays having reduced background noise"; USPN 5,681,697 to Urdea et al. entitled "Solution phase nucleic acid sandwich assays having reduced background noise and kits therefor"; USPN 5,124,246 to Urdea et al. entitled "Nucleic acid multimers and amplified nucleic acid hybridization assays using same"; USPN 5,624,802 to Urdea et al. entitled "Nucleic acid multimers and amplified nucleic acid hybridization assays using same"; USPN 5,849,481 to Urdea et al. entitled "Nucleic acid hybridization assays employing large comb-type branched polynucleotides"; USPN 5,710,264 to Urdea et al. entitled "Large comb type branched polynucleotides"; USPN 5,594,118 to Urdea and Horn entitled "Modified N-4 nucleotides for use in amplified nucleic acid hybridization assays"; USPN 5,093,232 to Urdea and Horn entitled "Nucleic acid probes"; USPN 4,910,300 to Urdea and Horn entitled "Method for making nucleic acid probes"; USPN 5,359,100; USPN 5,571,670; USPN 5,614,362; USPN 6,235,465; USPN 5,712,383; USPN 5,747,244; USPN 6,232,462; USPN 5,681,702; USPN 5,780,610; USPN 5,780,227 to Sheridan et al. entitled "Oligonucleotide probe conjugated to a purified hydrophilic alkaline phosphatase and uses thereof"; U.S. patent application Publication No. US2002172950 by Kenny et al. entitled "Highly sensitive gene detection and localization using in situ branched-DNA hybridization"; Wang et al. (1997) "Regulation of insulin preRNA splicing by glucose" Proc Nat Acad Sci USA 94:4360-4365; Collins et al. (1998) "Branched DNA (bDNA) technology for direct quantification of nucleic acids: Design and performance" in Gene Quantification, F Ferre, ed.; and Wilber and Urdea (1998) "Quantification of HCV RNA in clinical specimens by branched DNA (bDNA) technology" Methods in Molecular Medicine: Hepatitis C 19:71-78. In addition, kits for performing basic bDNA assays (QuantiGene^{TM} kits, comprising instructions and reagents such as amplification multimers, alkaline phosphatase labeled label probes, chemilumigenic substrate, capture probes immobilized on a solid support, and the like) are commercially available, e.g., from Panomics, Inc. (on the world wide web at (www.) panomics.com). Software for designing probe sets for a given mRNA target (i.e., for designing the regions of the CEs, LEs, and optionally BPs that are complementary to the target) is also commercially available (e.g., ProbeDesigner^{TM} from Panomics, Inc.; see also Bushnell et al. (1999) "ProbeDesigner: for the design of probe sets for branched DNA (bDNA) signal amplification assays Bioinformatics 15:348-55).

[0073] The basic bDNA assay, however, permits detection of only a single target nucleic acid per assay, while, as described above, detection of multiple nucleic acids is frequently desirable.

[0074] Among other aspects, the present invention provides a multiplex bDNA assay that can be used for simultaneous detection of 10 or more target nucleic acids. The assay temperature and the stability of the complex between a single CE and its corresponding CP can be controlled such that binding of a single CE to a nucleic acid and to the CP is not sufficient to stably capture the nucleic acid on the surface to which the CP is bound, whereas simultaneous binding of two or more CEs to a nucleic acid can capture it on the surface. Requiring such cooperative hybridization of multiple CEs for capture of each nucleic acid of interest results in high specificity and low background from cross-hybridization of the CEs with other, non-target nucleic acids. For an assay to achieve high specificity and sensitivity, it preferably has a low background, resulting, e.g., from minimal cross-hybridization. Such low background and minimal cross-hybridization are typically substantially more difficult to achieve in a multiplex assay than a single-plex assay, because the number of potential nonspecific interactions are greatly increased in a multiplex assay due to the increased number of probes used in the assay (e.g., the greater number of CEs and LEs). Requiring multiple simultaneous CE-CP interactions for the capture of a target nucleic acid minimizes the chance that nonspecific capture will occur, even when some nonspecific CE-CP interactions do occur.

[0075] The methods of the invention can be used for multiplex detection of 10 or more nucleic acids simultaneously, for example, from even complex samples, without requiring prior purification of the nucleic acids, when the nucleic acids are present at low concentration, and/or in the presence of other, highly similar nucleic acids. In one aspect, the methods involve capture of the nucleic acids to particles (e.g., distinguishable subsets of microspheres), while in another aspect, the nucleic acids are captured to a spatially addressable solid support. Compositions and related to the methods are also provided.

METHODS

[0076] As noted, one aspect of the invention provides multiplex bDNA assays. Thus, one general class of embodiments includes methods of detecting 10 or more nucleic acids of interest. In the methods, a sample, a pooled population of particles, and 10 or more subsets of n capture extenders, wherein n is at least two, are provided. The sample comprises or is suspected of comprising the nucleic acids of interest. The pooled population of particles includes 10 or more subsets of particles, and a plurality of the particles in each subset are distinguishable from a plurality of the particles in every other subset. (Typically, substantially all of the particles in each subset are distinguishable from substantially all of the particles in every other subset.) The particles in each subset have associated therewith a different capture probe. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected subset of the particles. Each nucleic acid of interest can thus, by hybridizing to its corresponding subset of n capture extenders which are in turn hybridized to a corresponding capture probe, be associated with an identifiable subset of the particles.

[0077] The sample, the pooled population of particles, and the subsets of n capture extenders are contacted, any nucleic acid of interest present in the sample is hybridized to its corresponding subset of n capture extenders, and the subset of n capture extenders is hybridized to its corresponding capture probe. The hybridizing the nucleic acid of interest to the n capture extenders and the n capture extenders to the corresponding capture probe captures the nucleic acid on the subset of particles with which the capture extenders are associated. The hybridizing the subset of n capture extenders to the corresponding capture probe is performed at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and its corresponding capture probe. Binding of a single capture extender to its corresponding nucleic acid (or to an extraneous nucleic acid) and capture probe is thus typically insufficient to capture the nucleic acid on the corresponding subset of particles. It will be evident that the hybridization temperature is typically less than a T_m of a complex between the nucleic acid of interest, all n corresponding capture extenders, and the corresponding capture probe.

[0078] To determine which subsets of particles have a nucleic acid of interest captured on the particles, one or more (e.g., two or more) label extenders and a label probe system comprising a label are hybridized to any nucleic acid of interest captured on the particles, and at least a portion of the particles from each subset are identified and the presence or absence of the label is detected on those particles. Since a correlation exists between a particular subset of particles and a particular nucleic acid of interest, which subsets of particles have the label present indicates which of the nucleic acids of interest were present in the sample.

[0079] The methods are useful for multiplex detection of nucleic acids, optionally highly multiplex detection. Thus, the two or more nucleic acids of interest (i.e., the nucleic acids to be detected) optionally comprise five or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or even 100 or more nucleic acids of interest. A like number of subsets of particles and subsets of CEs are provided; thus, the two or more subsets of particles can comprise five or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or even 100 or more subsets of particles, while the two or more subsets of n capture extenders can comprise five or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or even 100 or more subsets of n capture extenders.

[0080] Essentially any suitable particles, e.g., particles having distinguishable characteristics and to which capture probes can be attached, can be used. For example, in one preferred class of embodiments, the particles are microspheres. The microspheres of each subset can be distinguishable from those of the other subsets, e.g., on the basis of their fluorescent emission spectrum, their diameter, or a combination thereof. For example, the microspheres of each subset can be labeled with a unique fluorescent dye or mixture of such dyes, quantum dots with distinguishable emission spectra, and/or the like. As another example, the particles of each subset can be identified by an optical barcode, unique to that subset, present on the particles.

[0081] The particles optionally have additional desirable characteristics. For example, the particles can be magnetic or paramagnetic, which provides a convenient means for separating the particles from solution, e.g., to simplify separation of the particles from any materials not bound to the particles.

[0082] As noted, each of the 10 or more subsets of capture extenders includes n capture extenders, where n is at least two. Preferably, n is at least three, and n can be at least four or at least five or more. Typically, but not necessarily, n is at most ten. For example, n can be between three and ten, e.g., between five and ten or between five and seven, inclusive. Use of fewer capture extenders can be advantageous, for example, in embodiments in which nucleic acids of interest are to be specifically detected from samples including other nucleic acids with sequences very similar to that of the nucleic acids of interest. In other embodiments (e.g., embodiments in which capture of as much of the nucleic acid as possible is desired), however, n can be more than 10, e.g., between 20 and 50. n can be the same for all of the subsets of capture extenders, but it need not be; for example, one subset can include three capture extenders while another subset includes five capture extenders. The n capture extenders in a subset preferably hybridize to nonoverlapping polynucleotide sequences in the corresponding nucleic acid of interest. The nonoverlapping polynucleotide sequences can, but need not be, consecutive within the nucleic acid of interest.

[0083] Each capture extender is capable of hybridizing to its corresponding capture probe. The capture extender typically includes a polynucleotide sequence C-1 that is complementary to a polynucleotide sequence C-2 in its corresponding capture probe. In one aspect, C-1 and C-2 are 20 nucleotides or less in length. In one class of embodiments, C-1 and C-2 are between 9 and 17 nucleotides in length (inclusive), preferably between 12 and 15 nucleotides (inclusive). For example, C-1 and C-2 can be 14, 15, 16, or 17 nucleotides in length, or they can be between 9 and 13 nucleotides in length (e.g., for lower hybridization temperatures, e.g., hybridization at room temperature).

[0084] The capture probe can include polynucleotide sequence in addition to C-2, or C-2 can comprise the entire polynucleotide sequence of the capture probe. For example, each capture probe optionally includes a linker sequence between the site of attachment of the capture probe to the particles and sequence C-2 (e.g., a linker sequence containing 8 Ts, as just one possible example).

[0085] It will be evident that the amount of overlap between each individual capture extender and its corresponding capture probe (i.e., the length of C-1 and C-2) affects the T_m of the complex between that capture extender and capture probe, as does, e.g., the GC base content of sequences C-1 and C-2. Typically, all the capture probes are the same length (as are sequences C-1 and C-2) from subset of particles to subset. However, depending, e.g., on the precise nucleotide sequence of C-2, different capture probes optionally have different lengths and/or different length sequences C-2, to achieve the desired T_m. Different capture probe-capture extender complexes optionally have the same or different T_ms.

[0086] It will also be evident that the number of capture extenders required for stable capture of a nucleic acid depends, in part, on the amount of overlap between the capture extenders and the capture probe (i.e., the length of C-1 and C-2). For example, if n is 5-7 for a 14 nucleotide overlap, n could be 3-5 for a 15 nucleotide overlap or 2-3 for a 16 nucleotide overlap.

[0087] As noted, the hybridizing the subset of n capture extenders to the corresponding capture probe is performed at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and its corresponding capture probe. The hybridization temperature is typically about 5°C or more greater than the T_m, e.g., about 7°C or more, about 10°C or more, about 12°C or more, about 15°C or more, about 17°C or more, or even about 20°C or more greater than the T_m.

[0088] Stable capture of nucleic acids of interest, e.g., while minimizing capture of extraneous nucleic acids (e.g., those to which n-1 or fewer of the capture extenders bind) can be achieved, for example, by balancing n (the number of capture extenders), the amount of overlap between the capture extenders and the capture probe (the length of C-1 and C-2), and/or the stringency of the conditions under which the capture extenders, the nucleic acids, and the capture probes are hybridized.

[0089] Appropriate combinations of n, amount of complementarity between the capture extenders and the capture probe, and stringency of hybridization can, for example, be determine experimentally by one of skill in the art. For example, as illustrated in the experiments described in Example 1 herein, a particular value of n and a particular set of hybridization conditions can be selected, while the number of nucleotides of complementarity between the capture extenders and the capture probe is varied until hybridization of the n capture extenders to a nucleic acid captures the nucleic acid while hybridization of a single capture extender does not efficiently capture the nucleic acid. The experiments described in Example 1 also illustrate selection of n, amount of complementarity, and stringency of hybridization such that the desired nucleic acid of interest is captured while other nucleic acids present in the sample are not efficiently captured. Stringency can be controlled, for example, by controlling the formamide concentration, chaotropic salt concentration, salt concentration, pH, organic solvent content, and/or hybridization temperature.

[0090] As noted, the T_m of any nucleic acid duplex can be directly measured, using techniques well known in the art. For example, a thermal denaturation curve can be obtained for the duplex, the midpoint of which corresponds to the T_m. It will be evident that such denaturation curves can be obtained under conditions having essentially any relevant pH, salt concentration, solvent content, and/or the like.

[0091] The T_m for a particular duplex (e.g., an approximate T_m) can also be calculated. For example, the T_m for an oligonucleotide-target duplex can be estimated using the following algorithm, which incorporates nearest neighbor thermodynamic parameters: Tm (Kelvin) = $\Delta H^\circ/(\Delta S^\circ + R \ln C_t)$, where the changes in standard enthalpy (ΔH°) and entropy (ΔS°) are calculated from nearest neighbor thermodynamic parameters (see, e.g., SantaLucia (1998) "A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics" Proc. Natl. Acad. Sci. USA 95: 1460-1465, Sugimoto et al. (1996) "Improved thermodynamic parameters and helix initiation factor to predict stability of DNA duplexes" Nucleic Acids Research 24: 4501-4505, Sugimoto et al. (1995) "Thermodynamic parameters to predict stability of RNA/DNA hybrid duplexes" Biochemistry 34:11211-11216, and et al. (1998) "Thermodynamic parameters for an expanded nearest-neighbor model for formation of RNA duplexes with Watson-Crick base pairs" Biochemistry 37: 14719-14735), R is the ideal gas constant (1.987 $\text{cal}\cdot\text{K}^{-1}\text{mole}^{-1}$), and C_t is the molar concentration of the oligonucleotide. The calculated T_m is optionally corrected for salt concentration, e.g., Na^+ concentration, using the formula $1/T_m(Na^+) = 1/T_m(1M) + (4.29f(G\cdot C)-3.95)x10^{-5} \ln[Na^+] + 9.40x10^{-6} \ln^2[Na^+]$. See, e.g., Owczarzy et al. (2004) "Effects of Sodium Ions on DNA Duplex Oligomers: Improved Predictions of Melting Temperatures" Biochemistry 43:3537-3554 for further details. A Web calculator for estimating Tm using the above algorithms is available on the Internet at scitools.idtdna.com/analyzer/oligocalc.asp. Other algorithms for calculating T_m are known in the art and are optionally applied to the present invention.

[0092] In one class of embodiments, contacting the sample, the pooled population of particles, and the subsets of n capture extenders comprises combining the sample with the subsets of n capture extenders to form a mixture, and then combining the mixture with the pooled population of particles. In this class of embodiments, the capture extenders typically hybridize first to the corresponding nucleic acid of interest and then to the corresponding particle-associated capture probe. The hybridizations can, however, occur simultaneously or even in the opposite order. Thus, in another exemplary class of embodiments, contacting the sample, the pooled population of particles, and the subsets of n capture extenders comprises combining the sample, the subsets of capture extenders, and the pooled population of particles.

[0093] In a preferred class of embodiments, hybridizing one or more label extenders and a label probe system to any nucleic acid of interest captured on the particles comprises providing two or more subsets of one or more label extenders, wherein each subset of label extenders is capable of hybridizing to one of the nucleic acids of interest, hybridizing any nucleic acid of interest captured on the particles to its corresponding subset of label extenders, and hybridizing the label probe system to the label extenders. The two or more subsets of label extenders can include, e.g., five or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or even 100 or more subsets of label extenders. Typically, each subset includes two or more label extenders. The hybridizations can be performed simultaneously or sequentially, in essentially any order. As just one example, the capture extenders and label extenders can be hybridized to their corresponding nucleic acids of interest, then the capture extenders can be hybridized to their corresponding capture probes, and then the label probe system can be hybridized to the label extenders.

[0094] The label probe system optionally includes an amplification multimer and a plurality of label probes, wherein the amplification multimer is capable of hybridizing to a label extender and to a plurality of label probes. In another aspect, the label probe system includes a preamplifier, a plurality of amplification multimers, and a plurality of label probes, wherein the preamplifier hybridizes to the label extenders, and the amplification multimers hybridize to the preamplifier and to the plurality of label probes. As another example, the label probe system can include only label probes, which hybridize directly to the label extenders. In one class of embodiments, the label probe comprises the label. In other embodiments, the label probe is configured to bind a label; for example, a biotinylated label probe can bind to a streptavidin-associated label.

[0095] The label can be essentially any convenient label that directly or indirectly provides a detectable signal. In one aspect, the label is a fluorescent label (e.g., a fluorophore or quantum dot). Detecting the presence of the label on the particles thus comprises detecting a fluorescent signal from the label. Fluorescent emission by the label is typically distinguishable from any fluorescent emission by the particles, e.g., microspheres, and many suitable fluorescent label-fluorescent microsphere combinations are possible. As other examples, the label can be a luminescent label, a light-scattering label (e.g., colloidal gold particles), or an enzyme (e.g., HRP).

[0096] The methods can optionally be used to quantitate the amounts of the nucleic acids of interest present in the sample. For example, in one class of embodiments, an intensity of a signal from the label is measured, e.g., for each subset of particles, and correlated with a quantity of the corresponding nucleic acid of interest present.

[0097] As noted, blocking probes are optionally also hybridized to the nucleic acids of interest, which can reduce background in the assay. For a given nucleic acid of interest, the corresponding capture extenders, label extenders, and optional blocking probes are preferably complementary to physically distinct, nonoverlapping sequences in the nucleic acid of interest, which are preferably, but not necessarily, contiguous. The T_ms of the capture extender-nucleic acid, label extender-nucleic acid, and blocking probe-nucleic acid complexes are preferably greater than the hybridization temperature, e.g., by 5°C or 10°C or preferably by 15°C or more, such that these complexes are stable at the hybridization temperature. Potential CE and LE sequences (e.g., potential sequences C-3. and L-1) are optionally examined for possible interactions with non-corresponding nucleic acids of interest, LEs or CEs, the amplification multimer, the preamplifier, the label probe, and/or any relevant genomic sequences, for example; sequences expected to cross-hybridize with undesired nucleic acids are typically not selected for use in the CEs or LEs. See, e.g., Example 1 herein and Player et al. (2001) " Single-copy gene detection using branched DNA (bDNA) in situ hybridization" J Histochem Cytochem 49: 603-611. Examination can be, e.g., visual (e.g., visual examination for complementarity), computational (e.g., computation and comparison of binding free energies), and/or experimental (e.g., cross-hybridization experiments). Capture probe sequences are preferably similarly examined, to ensure that the polynucleotide sequence C-1 complementary to a particular capture probe's sequence C-2 is not expected to cross-hybridize with any of the other capture probes that are to be associated with other subsets of particles. See, e.g., Example 1 herein.

[0098] At any of various steps, materials not captured on the particles are optionally separated from the particles. For example, after the capture extenders, nucleic acids, label extenders, blocking probes, and particle-bound capture probes are hybridized, the particles are optionally washed to remove unbound nucleic acids and probes; after the label extenders and amplification multimer are hybridized, the particles are optionally washed to remove unbound amplification multimer; and/or after the label probes are hybridized to the amplification multimer, the particles are optionally washed to remove unbound label probe prior to detection of the label.

[0099] One or more of the subsets of particles is optionally isolated, whereby the associated nucleic acid of interest is isolated. The isolated nucleic acid can optionally be removed from the particles and/or subjected to further manipulation, if desired (e.g., amplification by PCR or the like).

[0100] An exemplary embodiment is schematically illustrated in **Figure 2**. **Panel A** illustrates three distinguishable subsets of microspheres **201**, **202**, and **203**, which have associated therewith capture probes **204**, **205**, and **206**, respectively. Each capture probe includes a sequence C-2 (**250**), which is different from subset to subset of microspheres. The three subsets of microspheres are combined to form pooled population **208** (**Panel B**). A subset of three capture extenders is provided for each nucleic acid of interest; subset **211** for nucleic acid **214**, subset **212** for nucleic acid **215** which is not present, and subset **213** for nucleic acid **216**. Each capture extender includes sequences C-1 (**251**, complementary to the respective capture probe's sequence C-2) and C-3 (**252**, complementary to a sequence in the corresponding nucleic acid of interest). Three subsets of label extenders (**221**, **222**, and **223** for nucleic acids **214**, **215**, and **216**, respectively) and three subsets of blocking probes (**224**, **225**, and **226** for nucleic acids **214**, **215**, and **216**, respectively) are also provided. Each label extender includes sequences L-1 (**254**, complementary to a sequence in the corresponding nucleic acid of interest) and L-2 (**255**, complementary to M-1). Non-target nucleic acids **230** are also present in the sample of nucleic acids.

[0101] Nucleic acids **214** and **216** are hybridized to their corresponding subset of capture extenders (**211** and **213**, respectively), and the capture extenders are hybridized to the corresponding capture probes (**204** and **206**, respectively), capturing nucleic acids **214** and **216** on microspheres **201** and **203**, respectively (**Panel C**). Materials not bound to the microspheres (e.g., capture extenders **212**, nucleic acids **230**, etc.) are separated from the microspheres by washing. Label probe system **240** including amplification multimer **241** (which includes sequences M-1 **257** and M-2 **258**) and label probe **242** (which contains label **243**) is hybridized to label extenders **221** and **223**, which are hybridized to nucleic acids **214** and **216**, respectively (**Panel D**). Materials not captured on the microspheres are optionally removed by washing the microspheres. Microspheres from each subset are identified, e.g., by their fluorescent emission spectrum (λ_2 and λ_3, **Panel E**), and the presence or absence of the label on each subset of microspheres is detected (λ_1, **Panel E**). Since each nucleic acid of interest is associated with a distinct subset of microspheres, the presence of the label on a given subset of microspheres correlates with the presence of the corresponding nucleic acid in the original sample.

[0102] As depicted in **Figure 2**, all of the label extenders in all of the subsets typically include an identical sequence L-2. Optionally, however, different label extenders (e.g., label extenders in different subsets) can include different sequences L-2. Also as depicted in **Figure 2**, each capture probe typically includes a single sequence C-2 and thus hybridizes to a single capture extender. Optionally, however, a capture probe can include two or more sequences C-2 and hybridize to two or more capture extenders. Similarly, as depicted, each of the capture extenders in a particular subset typically includes an identical sequence C-1, and thus only a single capture probe is needed for each subset of particles; however, different capture extenders within a subset optionally include different sequences C-1 (and thus hybridize to different sequences C-2, within a single capture probe or different capture probes on the surface of the corresponding subset of particles).

[0103] The methods can be used to detect the presence of the nucleic acids of interest in essentially any type of sample. For example, the sample can be derived from an animal, a human, a plant, a cultured cell, a virus, a bacterium, a pathogen, and/or a microorganism. The sample optionally includes a cell lysate, an intercellular fluid, a bodily fluid (including, but not limited to, blood, serum, saliva, urine, sputum, or spinal fluid), and/or a conditioned culture medium, and is optionally derived from a tissue (e.g., a tissue homogenate), a biopsy, and/or a tumor. Similarly, the nucleic acids can be essentially any desired nucleic acids. As just a few examples, the nucleic acids of interest can be derived from one or more of an animal, a human, a plant, a cultured cell, a microorganism, a virus, a bacterium, or a pathogen.

[0104] As noted, the methods can be used for gene expression analysis. Accordingly, in one class of embodiments, the two or more nucleic acids of interest comprise two or more mRNAs. The methods can also be used for clinical diagnosis and/or detection of microorganisms, e.g., pathogens. Thus, in certain embodiments, the nucleic acids include bacterial and/or viral genomic RNA and/or DNA (double-stranded or single-stranded), plasmid or other extra-genomic DNA, or other nucleic acids derived from microorganisms (pathogenic or otherwise). It will be evident that double-stranded nucleic acids of interest will typically be denatured before hybridization with capture extenders, label extenders, and the like.

[0105] Due to cooperative hybridization of multiple capture extenders to a nucleic acid of interest, for example, even nucleic acids present at low concentration can be captured and detected. Thus, in one class of embodiments, at least one of the nucleic acids of interest is present in the sample in a non-zero amount of 200 amol or less, 150 amol or less, 100 amol or less, 50 amol or less, 10 amol or less, 1 amol or less, or even 0.1 amol or less, 0.01 amol or less, 0.001 amol or less, or 0.0001 amol or less. Similarly, two nucleic acids of interest can be captured and detected simultaneously, even when they differ in concentration by 1000-fold or more in the sample. The methods are thus extremely versatile.

[0106] Capture of a particular nucleic acid is optionally quantitative. Thus, in one exemplary class of embodiments, the sample includes a first nucleic acid of interest, and at least 30%, at least 50%, at least 80%, at least 90%, at least 95%, or even at least 99% of a total amount of the first nucleic acid present in the sample is captured on a first subset of particles. Second, third, etc. nucleic acids can similarly be quantitatively captured. Such quantitative capture can occur without capture of a significant amount of undesired nucleic acids, even those of very similar sequence to the nucleic acid of interest.

[0107] Thus, in one class of embodiments, the sample comprises or is suspected of comprising a first nucleic acid of interest and a second nucleic acid which has a polynucleotide sequence which is 95% or more identical to that of the first nucleic acid (e.g., 96% or more, 97% or more, 98% or more, or even 99% or more identical). The first nucleic acid, if present in the sample, is captured on a first subset of particles, while the second nucleic acid comprises 1% or less of a total amount of nucleic acid captured on the first subset of particles (e.g., 0.5% or less, 0.2% or less, or even 0.1% or less). The second nucleic acid can be another nucleic acid of interest or simply any nucleic acid. Typically, capture extenders are chosen that hybridize to regions of the first nucleic acid having the greatest sequence difference from the second nucleic acid.

[0108] As just one example of how closely related nucleic acids can be distinguished using the methods of the invention, different splice variants of a given mRNA can be distinguished. Thus, in one class of embodiments, the sample comprises a first nucleic acid of interest and a second nucleic acid, where the first nucleic acid is a first splice variant and the second nucleic acid is a second splice variant of the given mRNA. A first subset of n capture extenders is capable of hybridizing to the first splice variant, of which at most n-1 capture extenders are capable of hybridizing to the second splice variant. Optionally, at least 80% or more, 90% or more, or 95% or more of the first splice variant is captured on a first subset of particles while at most 10% or less, 5% or less, 3% or less, or 1% or less of the second splice variant is captured on the first subset of particles. Preferably, hybridization of the n capture extenders to the first splice variant captures the first splice variant on a first subset of particles while hybridization of the at most n-1 capture extenders to the second splice variant does not capture the second splice variant on the first subset of particles.

[0109] An exemplary embodiment illustrating detection of two splice variants is schematically depicted in **Figure 3.** In this example, three CEs **11** hybridize to first splice variant **21,** one to each exon **(24** and **26)** and one to splice junction **27** (the only sequence found in first splice variant **21** and not also found in second splice variant **22);** two of these bind to second splice variant **22.** Similarly, three CEs **12** bind to second splice variant **22,** one to intron **25** and one to each of the splice junctions; none of these bind to first splice variant **21.** Through cooperative hybridization of the CEs to the splice variants and to the corresponding capture probes **(4** and **5),** splice variants **21** and **22** are each captured specifically only on the corresponding subset of microspheres (**1** and **2,** respectively). LEs **14** and **15** are hybridized with splice variants **21** and **22,** respectively, and BPs **13** are hybridized with first splice variant **21.** Amplification multimers **30** are then hybridized to the LEs, and label probes **31** are hybridized to the amplification multimers. Microspheres from the two subsets are then identified, and the presence or absence of the label on the microspheres is detected and correlated with the presence or absence of the corresponding splice variant. For additional details on detection of splice variants, see U.S. patent application 60/501,598, filed September 9, 2003, by Yuling Luo, entitled "Methods of capturing, detecting, and quantitating splice variants." Optionally, for any nucleic acid, hybridization of a first subset of n capture extenders to a first nucleic acid captures the first nucleic acid on a first subset of particles while hybridization of at most n-1 of the capture extenders to a second nucleic acid does not capture the second nucleic acid on the first subset of particles.

[0110] It will be evident that nucleic acids that do not have 100% identical sequences are alternatively optionally captured on the same subset of particles, if desired. For example, a first and a second nucleic acid are optionally both captured on a first subset of particles, through binding of the same or different subsets of capture extenders. The first and second nucleic acids can be closely related; for example, splice variants of a particular mRNA, different alleles of a gene, somatic mutations, homologs, or the like.

[0111] A capture probe and/or capture extender optionally comprises at least one non-natural nucleotide. For example, a capture probe and the corresponding capture extender optionally comprise, at complementary positions, at least one pair of non-natural nucleotides that base pair with each other but that do not Watson-Crick base pair with the bases typical to biological DNA or RNA (i.e., A, C, G, T, or U). Examples of nonnatural nucleotides include, but are not limited to, Locked NucleicAcid™ nucleotides (available from Exiqon A/S, (www.) exiqon.com; see, e.g., SantaLucia Jr. (1998) Proc Natl Acad Sci 95:1460-1465) and isoG, isoC, and other nucleotides used in the AEGIS system (Artificially Expanded Genetic Information System, available from EraGen Biosciences, (www.) eragen.com; see, e.g., USPN 6,001,983, USPN 6,037,120, and USPN 6,140,496). Use of such non-natural base pairs (e.g., isoG-isoC base pairs) in the capture probes and capture extenders can, for example, reduce background and/or simplify probe design by decreasing cross hybridization, or it can permit use of shorter CPs and CEs when the non-natural base pairs have higher binding affinities than do natural base pairs. (Non-natural nucleotides can similarly be included in the label extenders, preamplifiers, amplification multimers, and/or label probes, if desired.)

[0112] The preceding embodiments include capture of the nucleic acids of interest on particles. Alternatively, the nucleic acids can be captured at different positions on a non-particulate, spatially addressable solid support. Accordingly, another general class of embodiments includes methods of detecting 10 or more nucleic acids of interest. In the methods, a sample, a solid support, and 10 or more subsets of n capture extenders, wherein n is at least two, are provided. The sample comprises or is suspected of comprising the nucleic acids of interest. The solid support comprises 10 or more capture probes, each of which is provided at a selected position on the solid support. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected position on the solid support. Each nucleic acid of interest can thus, by hybridizing to its corresponding subset of n capture extenders which are in turn hybridized to a corresponding capture probe, be associated with, e.g., a known, predetermined location on the solid support. The sample, the solid support, and the subsets of n capture extenders are contacted, any nucleic acid of interest present in the sample is hybridized to its corresponding subset of n capture extenders, and the subset of n capture extenders is hybridized to its corresponding capture probe. The hybridizing the nucleic acid of interest to the n capture extenders and the n capture extenders to the corresponding capture probe captures the nucleic acid on the solid support at the selected position with which the capture extenders are associated. To determine which positions on the solid support have a nucleic acid of interest captured at that position, one or more (e.g., two or more) label extenders and a label probe system comprising a label are hybridized to any nucleic acid of interest captured on the solid support, and the presence or absence of the label at the selected positions on the solid support is detected. Since a correlation exists between a particular position on the support and a particular nucleic acid of interest, which positions have a label present indicates which of the nucleic acids of interest were present in the sample.

[0113] The hybridizing the subset of n capture extenders to the corresponding capture probe is typically performed at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and its corresponding capture probe. For example, the hybridization temperature can be about 5°C or more greater than the T_m, e.g., about 7°C or more, about 10°C or more, about 12°C or more, about 15°C or more, about 17°C or more, or even about 20°C or more greater than the T_m.

[0114] The methods are useful for multiplex detection of nucleic acids, optionally highly multiplex detection. Thus, the two or more nucleic acids of interest (i.e., the nucleic acids to be detected) optionally comprise five or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, 100 or more, 10^3 or more, or 10^4 or more nucleic acids of interest. A like number of selected positions on the solid support and subsets of CEs are provided; thus, the two or more selected positions can comprise five or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, 100 or more, 10^3 or more, or 10^4 or more selected positions, while the two or more subsets of n capture extenders can comprise five or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, 100 or more, 10^3 or more, or 10^4 or more subsets of n capture extenders.

[0115] The solid support typically has a planar surface and is typically rigid, but essentially any spatially addressable solid support can be adapted to the practice of the present invention. Exemplary materials for the solid support include, but are not limited to, glass, silicon, silica, quartz, plastic, polystyrene, nylon, and nitrocellulose. As just one examples, an array of capture probes can be formed at selected positions on a glass slide as the solid support.

[0116] Essentially all of the features noted for the methods above apply to these embodiments as well, as relevant; for example, with respect to number of capture extenders per subset, composition of the label probe system, type of label, use of blocking probes, configuration of the capture extenders, capture probes, label extenders, and/or blocking probes, quantitation of the nucleic acids of interest, source of the sample and/or nucleic acids, and/or the like.

[0117] For example, in one class of embodiments, contacting the sample, the solid support, and the subsets of n capture extenders comprises combining the sample with the subsets of n capture extenders to form a mixture, and then contacting the mixture with the solid support. In this class of embodiments, the capture extenders typically hybridize first to the corresponding nucleic acid of interest and then to the corresponding particle-associated capture probe. In other embodiments, however, the hybridizations can occur simultaneously or even in the opposite order.

[0118] At any of various steps, materials not captured on the solid support are optionally separated from the solid support. For example, after the capture extenders, nucleic acids, label extenders, blocking probes, and support-bound capture probes are hybridized, the solid support is optionally washed to remove unbound nucleic acids and probes; after the label extenders and amplification multimer are hybridized, the support is optionally washed to remove unbound amplification multimer; and/or after the label probes are hybridized to the amplification multimer, the support is optionally washed to remove unbound label probe prior to detection of the label.

[0119] As for the embodiments described above, capture of a particular nucleic acid is optionally quantitative. Thus, in one exemplary class of embodiments, the sample includes a first nucleic acid of interest, and at least 30%, at least 50%, at least 80%, at least 90%, at least 95%, or even at least 99% of a total amount of the first nucleic acid present in the sample is captured at a first selected position on the solid support. Second, third, etc. nucleic acids can similarly be quantitatively captured. Such quantitative capture can occur without capture of a significant amount of undesired nucleic acids, even those of very similar sequence to the nucleic acid of interest.

[0120] Thus, in one class of embodiments, the sample comprises or is suspected of comprising a first nucleic acid of interest and a second nucleic acid which has a polynucleotide sequence which is 95% or more identical to that of the first nucleic acid (e.g., 96% or more, 97% or more, 98% or more, or even 99% or more identical). The first nucleic acid, if present in the sample, is captured at a first selected position on the solid support, while the second nucleic acid comprises 1% or less of a total amount of nucleic acid captured at the first position (e.g., 0.5% or less, 0.2% or less, or even 0.1% or less). The second nucleic acid can be another nucleic acid of interest or simply any nucleic acid. Typically, capture extenders are chosen that hybridize to regions of the first nucleic acid having the greatest sequence difference from the second nucleic acid.

[0121] As just one example of how closely related nucleic acids can be distinguished using the methods of the invention, different splice variants of a given mRNA can be distinguished. Thus, in one class of embodiments, the sample comprises a first nucleic acid of interest and a second nucleic acid, where the first nucleic acid is a first splice variant and the second nucleic acid is a second splice variant of the given mRNA. A first subset of n capture extenders is capable of hybridizing to the first splice variant, of which at most n-1 capture extenders are capable of hybridizing to the second splice variant. Optionally, at least 80% or more, 90% or more, or 95% or more of the first splice variant is captured at a first selected position on the solid support while at most 10% or less, 5% or less, 3% or less, or 1% or less of the second splice variant is captured at the first position. Preferably, hybridization of the n capture extenders to the first splice variant captures the first splice variant at a first selected position on the solid support while hybridization of the at most n-1 capture extenders to the second splice variant does not capture the second splice variant at the first position.

[0122] It will be evident that nucleic acids that do not have 100% identical sequences are alternatively optionally captured at the same position of the support, if desired. For example, a first and a second nucleic acid are optionally both captured at a first position, through binding of the same or different subsets of capture extenders. The first and second nucleic acids can be closely related; for example, splice variants of a particular mRNA, different alleles of a gene, somatic mutations, homologs, or the like.

[0123] An exemplary embodiment is schematically illustrated in **Figure 4. Panel A** depicts solid support **301** having nine capture probes provided on it at nine selected positions (e.g., **334-336**). **Panel B** depicts a cross section of solid support **301,** with distinct capture probes **304, 305,** and **306** at different selected positions on the support (**334, 335,** and **336,** respectively). A subset of capture extenders is provided for each nucleic acid of interest. Only three subsets are depicted; subset **311** for nucleic acid **314,** subset **312** for nucleic acid **315** which is not present, and subset **313** for nucleic acid **316.** Each capture extender includes sequences C-1 (**351,** complementary to the respective capture probe's sequence C-2) and C-3 (**352,** complementary to a sequence in the corresponding nucleic acid of interest). Three subsets of label extenders (**321, 322,** and **323** for nucleic acids **314, 315,** and **316,** respectively) and three subsets of blocking probes (**324, 325,** and **326** for nucleic acids **314, 315,** and **316,** respectively) are also depicted (although nine would typically be provided, one for each nucleic acid of interest). Each label extender includes sequences **L-1** (**354,** complementary to a sequence in the corresponding nucleic acid of interest) and **L-2** (**355,** complementary to **M-1**). Non-target nucleic acids **330** are also present in the sample of nucleic acids.

[0124] Nucleic acids **314** and **316** are hybridized to their corresponding subset of capture extenders (**311** and **313,** respectively), and the capture extenders are hybridized to the corresponding capture probes (**304** and **306,** respectively), capturing nucleic acids **314** and **316** at selected positions **334** and **336,** respectively (**Panel C**). Materials not bound to the solid support (e.g., capture extenders **312,** nucleic acids **330,** etc.) are separated from the support by washing. Label probe system **340** including amplification multimer **341** (which includes sequences **M-1 357** and **M-2 358**) and label probe **342** (which contains label **343**) is hybridized to label extenders **321** and **323,** which are hybridized to nucleic acids **314** and **316,** respectively (**Panel D**). Materials not captured on the solid support are optionally removed by washing the support, and the presence or absence of the label at each position on the solid support is detected. Since each nucleic acid of interest is associated with a distinct position on the support, the presence of the label at a given position on the support correlates with the presence of the corresponding nucleic acid in the original sample.

[0125] The methods of the present invention offer a number of advantages. For example, a single array of capture probes at selected positions on a solid support can be manufactured, and this single array can be used to detect essentially any desired group of nucleic acids of interest simply by synthesizing appropriate subsets of capture extenders, label extenders, and the like. A new array need not be manufactured for each new group of nucleic acids to be detected, unlike conventional microarray technologies in which arrays of target-specific probes attached to a solid support are utilized, necessitating the manufacture of a new array for each new group of target nucleic acids to be detected. Similarly, a single population of subsets of particles comprising capture probes can be manufactured and used for detection of essentially any desired group of nucleic acids of interest.

COMPOSITIONS

[0126] Compositions related to the methods are another feature of the invention. Thus, one general class of embodiments provides a composition for detecting 10 or more nucleic acids of interest, the composition that includes or more subsets of particles, 10 or more subsets of n capture extenders, wherein n is at least two, 10 or more subsets of one or more label extenders, and a label probe system comprising a label. A plurality of the particles in each subset are distinguishable from a plurality of the particles in every other subset. (Typically, substantially all of the particles in each subset are distinguishable from substantially all of the particles in every other subset.) The particles in each subset have associated therewith a different capture probe. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected subset of the particles. When the nucleic acid of interest corresponding to a subset of n capture extenders is present in the composition and is hybridized to the subset of n capture extenders, which are hybridized to the corresponding capture probe, the nucleic acid of interest is hybridized to the subset of n capture extenders at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and the capture probe.

[0127] The composition optionally includes a sample comprising or suspected of comprising at least one of the nucleic acids of interest, e.g., two or more, three or more, etc. nucleic acids. In one class of embodiments, the composition comprises one or more of the nucleic acids of interest. Each nucleic acid of interest is hybridized to its corresponding subset of n capture extenders, and the corresponding subset of n capture extenders is hybridized to its corresponding capture probe. Each nucleic acid of interest is thus associated with an identifiable subset of the particles. The composition is maintained at the hybridization temperature.

[0128] As noted, the hybridization temperature is greater than the T_m of each of the individual CE-CP complexes. The hybridization temperature is typically about 5°C or more greater than the T_m, e.g., about 7°C or more, about 10°C or more, about 12°C or more, about 15°C or more, about 17°C or more, or even about 20°C or more greater than the T_m.

[0129] Essentially all of the features noted for the methods above apply to these embodiments as well, as relevant; for example, with respect to number of capture extenders per subset, composition of the label probe system, type of label, inclusion of blocking probes, configuration of the capture extenders, capture probes, label extenders, and/or blocking probes, number of nucleic acids of interest and of subsets of particles, capture extenders and label extenders, type of particles, source of the sample and/or nucleic acids, and/or the like.

[0130] As noted, even nucleic acids present at low concentration can be captured. Thus, in one class of embodiments, at least one of the nucleic acids of interest is present in the composition in a non-zero amount of 200 amol or less, 150 amol or less, 100 amol or less, 50 amol or less, 10 amol or less, 1 amol or less, or even 0.1 amol or less, 0.01 amol or less, 0.001 amol or less, or 0.0001 amol or less. Similarly, two nucleic acids of interest can be captured simultaneously, even when they differ in concentration by 1000-fold or more in the composition.

[0131] Capture of a particular nucleic acid on the particles is optionally quantitative. Thus, in one exemplary class of embodiments, the composition includes a first nucleic acid of interest, and at least 30%, at least 50%, at least 80%, at least 90%, at least 95%, or even at least 99% of a total amount of the first nucleic acid present in the composition is captured on a first subset of particles. Second, third, etc. nucleic acids can similarly be quantitatively captured. Such quantitative capture can occur without capture of a significant amount of undesired nucleic acids, even those of very similar sequence to the nucleic acid of interest.

[0132] Thus, in one class of embodiments, the composition comprises or is suspected of comprising a first nucleic acid of interest and a second nucleic acid which has a polynucleotide sequence which is 95% or more identical to that of the first nucleic acid (e.g., 96% or more, 97% or more, 98% or more, or even 99% or more identical). The first nucleic acid, if present in the composition, is captured on a first subset of particles, while the second nucleic acid comprises 1% or less of a total amount of nucleic acid captured on the first subset of particles (e.g., 0.5% or less, 0.2% or less, or even 0.1% or less). The second nucleic acid can be another nucleic acid of interest or simply any nucleic acid. Typically, capture extenders are chosen that hybridize to regions of the first nucleic acid having the greatest sequence difference from the second nucleic acid.

[0133] In one exemplary class of embodiments in which related nucleic acids are differentially captured, the composition comprises a first nucleic acid of interest and a second nucleic acid, where the first nucleic acid is a first splice variant and the second nucleic acid is a second splice variant of a given mRNA. A first subset of n capture extenders is capable of hybridizing to the first splice variant, of which at most n-1 capture extenders are capable of hybridizing to the second splice variant. Optionally, at least 80% or more, 90% or more, or 95% or more of the first splice variant is captured on a first subset of particles while at most 10% or less, 5% or less, 3% or less, or 1% or less of the second splice variant is captured on the first subset of particles. Preferably, a first subset of n capture extenders is hybridized to the first splice variant, whereby the first splice variant is captured on a first subset of particles, and at most n-1 of the capture extenders are hybridized to the second splice variant, whereby the second splice variant is not captured on the first subset of particles.

[0134] A related general class of embodiments provides a composition comprising 10 or more subsets of particles, 10 or more subsets of n capture extenders, wherein n is at least two, 10 or more subsets of one or more label extenders, a label probe system comprising a label, and at least a first nucleic acid of interest. A plurality of the particles in each subset are distinguishable from a plurality of the particles in every other subset. (Typically, substantially all of the particles in each subset are distinguishable from substantially all of the particles in every other subset.) The particles in each subset have associated therewith a different capture probe. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected subset of the particles. In this class of embodiments, the composition is maintained at a hybridization temperature, which hybridization temperature is greater than a melting temperature T_m of a complex between each individual capture extender and its corresponding capture probe. The first nucleic acid of interest is hybridized to a first subset of n first capture extenders, which first capture extenders are hybridized to a first capture probe.

[0135] Essentially all of the features noted for the embodiments above apply to these embodiments as well, as relevant; for example, with respect to number of capture extenders per subset, composition of the label probe system, type of label, inclusion of blocking probes, configuration of the capture extenders, capture probes, label extenders, and/or blocking probes, number of nucleic acids of interest and of subsets of particles, capture extenders and label extenders, source of the sample and/or nucleic acids, and/or the like.

[0136] Another general class of embodiments provides a composition for detecting 10 or more nucleic acids of interest, the composition that includes a solid support comprising 10 or more capture probes, each of which is provided at a selected position on the solid support, 10 or more subsets of n capture extenders, wherein n is at least two, 10 or more subsets of one or more label extenders, and a label probe system comprising a label. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected position on the solid support.

[0137] The composition optionally includes a sample comprising or suspected of comprising at least one of the nucleic acids of interest, e.g., two or more, three or more, etc. nucleic acids. In one class of embodiments, the composition includes at least a first nucleic acid of interest and is maintained at a hybridization temperature. The first nucleic acid of interest is hybridized to a first subset of n first capture extenders, which first capture extenders are hybridized to a first capture probe; the first nucleic acid is thereby associated with a first selected position on the solid support. It will be evident that the composition optionally includes second, third, etc. nucleic acids of interest, which are likewise associated with second, third, etc. selected positions on the solid support through association with second, third, etc. subsets of capture extenders and second, third, etc. capture probes. The hybridization temperature is greater than a melting temperature T_m of a complex between each individual capture extender and its corresponding capture probe. The hybridization temperature is typically about 5°C or more greater than the T_m, e.g., about 7°C or more, about 10°C or more, about 12°C or more, about 15°C or more, about 17°C or more, or even about 20°C or more greater than the T_m.

[0138] Essentially all of the features noted for the embodiments above apply to these embodiments as well, as relevant; for example, with respect to number of capture extenders per subset, type of solid support, composition of the label probe system, type of label, inclusion of blocking probes, configuration of the capture extenders, capture probes, label extenders, and/or blocking probes, number of nucleic acids of interest and of selected positions on the solid support, capture extenders and label extenders, source of the sample and/or nucleic acids, and/or the like.

KITS

[0139] A kit for detecting two or more nucleic acids of interest is discussed herein. The kit includes two or more subsets of particles, two or more subsets of n capture extenders, wherein n is at least two, two or more subsets of one or more label extenders, and a label probe system comprising a label, packaged in one or more containers. A plurality of the particles in each subset are distinguishable from a plurality of the particles in every other subset. (Typically, substantially all of the particles in each subset are distinguishable from substantially all of the particles in every other subset.) The particles in each subset have associated therewith a different capture probe. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected subset of the particles. When the nucleic acid of interest corresponding to a subset of n capture extenders is hybridized to the subset of n capture extenders, which are hybridized to the corresponding capture probe, the nucleic acid of interest is hybridized to the subset of n capture extenders at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and the capture probe. The kit optionally also includes instructions for using the kit to capture and detect the nucleic acids of interest, one or more buffered solutions (e.g., lysis buffer, diluent, hybridization buffer, and/or wash buffer), standards comprising one or more nucleic acids at known concentration, and/or the like.

[0140] Essentially all of the features noted for the embodiments above apply to the kits as well, as relevant; for example, with respect to number of capture extenders per subset, composition of the label probe system, type of label, inclusion of blocking probes, configuration of the capture extenders, capture probes, label extenders, and/or blocking probes, number of nucleic acids of interest and of subsets of particles, capture extenders and label extenders, source of the sample and/or nucleic acids, and/or the like.

[0141] A related kit for detecting two or more nucleic acids of interest is discussed herein. The kit includes a solid support comprising two or more capture probes, each of which is provided at a selected position on the solid support, two or more subsets of n capture extenders, wherein n is at least two, two or more subsets of one or more label extenders, and a label probe system comprising a label, packaged in one or more containers. Each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected position on the solid support.

[0142] In one class of kits, when a nucleic acid of interest corresponding to a subset of n capture extenders is hybridized to the subset of n capture extenders, which are hybridized to the corresponding capture probe, the nucleic acid of interest is hybridized to the subset of n capture extenders at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and the capture probe. The hybridization temperature is typically about 5°C or more greater than the T_m, e.g., about 7°C or more, about 10°C or more, about 12°C or more, about 15°C or more, about 17°C or more, or even about 20°C or more greater than the T_m.

[0143] The kit optionally also includes instructions for using the kit to capture and detect the nucleic acids of interest, one or more buffered solutions (e.g., lysis buffer, diluent, hybridization buffer, and/or wash buffer), standards comprising one or more nucleic acids at known concentration, and/or the like.

[0144] Essentially all of the features noted for the embodiments above apply to these kits as well, as relevant; for example, with respect to number of capture extenders per subset, composition of the label probe system, type of label, inclusion of blocking probes, configuration of the capture extenders, capture probes, label extenders, and/or blocking probes, number of nucleic acids of interest and of selected positions on the solid support, capture extenders and label extenders, source of the sample and/or nucleic acids, and/or the like.

SYSTEMS

[0145] Discussed herein are systems, e.g., systems used to practice the methods herein and/or comprising the compositions described herein. The system can include, e.g., a fluid and/or microsphere handling element, a fluid and/or microsphere containing element, a laser for exciting a fluorescent label and/or fluorescent microspheres, a detector for detecting light emissions from a chemiluminescent reaction or fluorescent emissions from a fluorescent label and/or fluorescent microspheres, and/or a robotic element that moves other components of the system from place to place as needed (e.g., a multiwell plate handling element). For example, in one class of embodiments, a composition of the invention is contained in a flow cytometer, a Luminex 100™ or HTS™ instrument, a microplate reader, a microarray reader, a luminometer, a colorimeter, or like instrument.

[0146] The system can optionally include a computer. The computer can include appropriate software for receiving user instructions, either in the form of user input into a set of parameter fields, e.g., in a GUI, or in the form of preprogrammed instructions, e.g., preprogrammed for a variety of different specific operations. The software optionally converts these instructions to appropriate language for controlling the operation of components of the system (e.g., for controlling a fluid handling element, robotic element and/or laser). The computer can also receive data from other components of the system, e.g., from a detector, and can interpret the data, provide it to a user in a human readable format, or use that data to initiate further operations, in accordance with any programming by the user.

LABELS

[0147] A wide variety of labels are well known in the art and can be adapted to the practice of the present invention. For example, luminescent labels and light-scattering labels (e.g., colloidal gold particles) have been described See, e.g., Csaki et al. (2002) "Gold nanoparticles as novel label for DNA diagnostics" Expert Rev Mol Diagn 2:187-93.

[0148] As another example, a number of fluorescent labels are well known in the art, including but not limited to, hydrophobic fluorophores (e.g., phycoerythrin, rhodamine, Alexa Fluor 488 and fluorescein), green fluorescent protein (GFP) and variants thereof (e.g., cyan fluorescent protein and yellow fluorescent protein), and quantum dots. See e.g., Haughland (2003) Handbook of Fluorescent Probes and Research Products, Ninth Edition or Web Edition, from Molecular Probes, Inc., or The Handbook: A Guide to Fluorescent Probes and Labeling Technologies, Tenth Edition or Web Edition

(2006) from Invitrogen (available on the world wide web at probes.invitrogen.com/handbook) for descriptions of fluorophores emitting at various different wavelengths (including tandem conjugates of fluorophores that can facilitate simultaneous excitation and detection of multiple labeled species). For use of quantum dots as labels for biomolecules, see e.g., Dubertret et al. (2002) Science 298:1759; Nature Biotechnology (2003) 21:41-46; and Nature Biotechnology (2003) 21:47-51.

[0149] Labels can be introduced to molecules, e.g. polynucleotides, during synthesis or by postsynthetic reactions by techniques established in the art; for example, kits for fluorescently labeling polynucleotides with various fluorophores are available from Molecular Probes, Inc. ((www.) molecularprobes.com), and fluorophore-containing phosphoramidites for use in nucleic acid synthesis are commercially available. Similarly, signals from the labels (e.g., absorption by and/or fluorescent emission from a fluorescent label) can be detected by essentially any method known in the art. For example, multicolor detection, detection of FRET, fluorescence polarization, and the like, are well known in the art.

MICROSPHERES

[0150] Microspheres are preferred particles in certain embodiments described herein since they are generally stable, are widely available in a range of materials, surface chemistries and uniform sizes, and can be fluorescently dyed. Microspheres can be distinguished from each other by identifying characteristics such as their size (diameter) and/or their fluorescent emission spectra, for example.

[0151] Luminex Corporation ((www.) luminexcorp.com), for example, offers 100 sets of uniform diameter polystyrene microspheres. The microspheres of each set are internally labeled with a distinct ratio of two fluorophores. A flow cytometer or other suitable instrument can thus be used to classify each individual microsphere according to its predefined fluorescent emission ratio. Fluorescently-coded microsphere sets are also available from a number of other suppliers, including Radix Biosolutions ((www.) radixbiosolutions.com) and Upstate Biotechnology ((www.) upstatebiotech.com). Alternatively, BD Biosciences ((www.) bd.com) and Bangs Laboratories, Inc. ((www.) bangslabs.com) offer microsphere sets distinguishable by a combination of fluorescence and size. As another example, microspheres can be distinguished on the basis of size alone, but fewer sets of such microspheres can be multiplexed in an assay because aggregates of smaller microspheres can be difficult to distinguish from larger microspheres.

[0152] Microspheres with a variety of surface chemistries are commercially available, from the above suppliers and others (e.g., see additional suppliers listed in Kellar and Iannone (2002) "Multiplexed microsphere-based flow cytometric assays" Experimental Hematology 30:1227-1237 and Fitzgerald (2001) "Assays by the score" The Scientist 15[11]:25). For example, microspheres with carboxyl, hydrazide or maleimide groups are available and permit covalent coupling of molecules (e.g., polynucleotide capture probes with free amine, carboxyl, aldehyde, sulfhydryl or other reactive groups) to the microspheres. As another example, microspheres with surface avidin or streptavidin are available and can bind biotinylated capture probes; similarly, microspheres coated with biotin are available for binding capture probes conjugated to avidin or streptavidin. In addition, services that couple a capture reagent of the customer's choice to microspheres are commercially available, e.g., from Radix Biosolutions ((www.) radixbiosolutions.com).

[0153] Protocols for using such commercially available microspheres (e.g., methods of covalently coupling polynucleotides to carboxylated microspheres for use as capture probes, methods of blocking reactive sites on the microsphere surface that are not occupied by the polynucleotides, methods of binding biotinylated polynucleotides to avidin-functionalized microspheres, and the like) are typically supplied with the microspheres and are readily utilized and/or adapted by one of skill. In addition, coupling of reagents to microspheres is well described in the literature. For example, see Yang et al. (2001) "BADGE, Beads Array for the Detection of Gene Expression, a high-throughput diagnostic bioassay" Genome Res. 11:1888-98; Fulton et al. (1997) "Advanced multiplexed analysis with the FlowMetrix™ system" Clinical Chemistry 43:1749-1756; Jones et al. (2002) "Multiplex assay for detection of strain-specific antibodies against the two variable regions of the G protein of respiratory syncytial virus" 9:633-638; Camilla et al. (2001) "Flow cytometric microsphere-based immunoassay: Analysis of secreted cytokines in whole-blood samples from asthmatics" Clinical and Diagnostic Laboratory Immunology 8:776-784; Martins (2002) "Development of internal controls for the Luminex instrument as part of a multiplexed seven-analyte viral respiratory antibody profile" Clinical and Diagnostic Laboratory Immunology 9:41-45; Kellar and Iannone (2002) "Multiplexed microsphere-based flow cytometric assays" Experimental Hematology 30:1227-1237; Oliver et al. (1998) "Multiplexed analysis of human cytokines by use of the FlowMetrix system" Clinical Chemistry 44:2057-2060; Gordon and McDade (1997) "Multiplexed quantification of human IgG, IgA, and IgM with the FlowMetrix™ system" Clinical Chemistry 43:1799-1801; USPN 5,981,180 entitled "Multiplexed analysis of clinical specimens apparatus and methods" to Chandler et al. (November 9, 1999); USPN 6,449,562 entitled "Multiplexed analysis of clinical specimens apparatus and methods" to Chandler et al. (September 10, 2002); and references therein.

[0154] Methods of analyzing microsphere populations (e.g. methods of identifying microsphere subsets by their size and/or fluorescence characteristics, methods of using size to distinguish microsphere aggregates from single uniformly sized microspheres and eliminate aggregates from the analysis, methods of detecting the presence or absence of a fluorescent label on the microsphere subset, and the like) are also well described in the literature. See, e.g., the above references.

[0155] Suitable instruments, software, and the like for analyzing microsphere populations to distinguish subsets of microspheres and to detect the presence or absence of a label (e.g., a fluorescently labeled label probe) on each subset are commercially available. For example, flow cytometers are widely available, e.g., from Becton-Dickinson ((www.) bd.com) and Beckman Coulter ((www.) beckman.com). Luminex 100™ and Luminex HTS™ systems (which use microfluidics to align the microspheres and two lasers to excite the microspheres and the label) are available from Luminex Corporation ((www.) luminexcorp.com); the similar Bio-Plex™ Protein Array System is available from Bio-Rad Laboratories, Inc. ((www.) bio-rad.com). A confocal microplate reader suitable for microsphere analysis, the FMAT™ System 8100, is available from Applied Biosystems ((www.) appliedbiosystems.com).

[0156] As another example of particles that can be adapted for use in the present invention, sets of microbeads that include optical barcodes are available from CyVera Corporation ((www.) cyvera.com). The optical barcodes are holographically inscribed digital codes that diffract a laser beam incident on the particles, producing an optical signature unique for each set of microbeads.

MOLECULAR BIOLOGICAL TECHNIQUES

[0157] In practicing the present invention, many conventional techniques in molecular biology, microbiology, and recombinant DNA technology are optionally used. These techniques are well known and are explained in, for example, Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, CA; Sambrook et al., Molecular Cloning - A Laboratory Manual (3rd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 2000 and Current Protocols in Molecular Biology, F.M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (supplemented through 2005). Other useful references, e.g. for cell isolation and culture (e.g., for subsequent nucleic acid or protein isolation) include Freshney (1994) Culture of Animal Cells, a Manual of Basic Technique, third edition, Wiley-Liss, New York and the references cited therein; Payne et al. (1992) Plant Cell and Tissue Culture in Liquid Systems John Wiley & Sons, Inc. New York, NY; Gamborg and Phillips (Eds.) (1995) Plant Cell, Tissue and Organ Culture; Fundamental Methods Springer Lab Manual, Springer-Verlag (Berlin Heidelberg New York) and Atlas and Parks (Eds.) The Handbook of Microbiological Media (1993) CRC Press, Boca Raton, FL.

Making Polynucleotides

[0158] Methods of making nucleic acids (e.g., by in vitro amplification, purification from cells, or chemical synthesis), methods for manipulating nucleic acids (e.g., by restriction enzyme digestion, ligation, etc.) and various vectors, cell lines and the like useful in manipulating and making nucleic acids are described in the above references. In addition, methods of making branched polynucleotides (e.g., amplification multimers) are described in USPN 5,635,352, USPN 5,124,246, USPN 5,710,264, and USPN 5,849,481, as well as in other references mentioned above.

[0159] In addition, essentially any polynucleotide (including, e.g., labeled or biotinylated polynucleotides) can be custom or standard ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company ((www.) mcrc.com), The Great American Gene Company ((www.) genco.com), ExpressGen Inc. ((www.) expressgen.com), Qiagen (oligos.qiagen.com) and many others.

[0160] A label, biotin, or other moiety can optionally be introduced to a polynucleotide, either during or after synthesis. For example, a biotin phosphoramidite can be incorporated during chemical synthesis of a polynucleotide. Alternatively, any nucleic acid can be biotinylated using techniques known in the art; suitable reagents are commercially available, e.g., from Pierce Biotechnology ((www.) piercenet.com). Similarly, any nucleic acid can be fluorescently labeled, for example, by using commercially available kits such as those from Molecular Probes, Inc. ((www.) molecularprobes.com) or Pierce Biotechnology ((www.) piercenet.com) or by incorporating a fluorescently labeled phosphoramidite during chemical synthesis of a polynucleotide.

ARRAYS

[0161] In an array of capture probes on a solid support (e.g., a membrane, a glass or plastic slide, a silicon or quartz chip, a plate, or other spatially addressable solid support), each capture probe is typically bound (e.g., electrostatically or covalently bound, directly or via a linker) to the support at a unique selected location. Methods of making, using, and analyzing such arrays (e.g., microarrays) are well known in the art. See, e.g., Baldi et al. (2002) DNA Microarrays and Gene Expression: From Experiments to Data Analysis and Modeling, Cambridge University Press; Beaucage (2001) "Strategies in the preparation of DNA oligonucleotide arrays for diagnostic applications" Curr Med Chem 8:1213-1244; Schena, ed. (2000) Microarray Biochip Technology, pp.19-38, Eaton Publishing; technical note "Agilent SurePrint Technology: Content centered microarray design enabling speed and flexibility" available on the web at chem.agilent.com/

temp/rad01539/00039489.pdf; and references therein. Arrays of pre-synthesized polynucleotides can be formed (e.g., printed), for example, using commercially available instruments such as a GMS 417 Arrayer (Affymetrix, Santa Clara, CA). Alternatively, the polynucleotides can be synthesized at the selected positions on the solid support; see, e.g., USPN 6,852,490 and USPN 6,306,643, each to Gentanlen and Chee entitled "Methods of using an array of pooled probes in genetic analysis."

[0162] Suitable solid supports are commercially readily available. For example, a variety of membranes (e.g., nylon, PVDF, and nitrocellulose membranes) are commercially available, e.g., from Sigma-Aldrich, Inc. ((www.) sigmaaldrich.com). As another example, surface-modified and pre-coated slides with a variety of surface chemistries are commercially available, e.g., from TeleChem International ((www.) arrayit.com), Corning, Inc. (Corning, NY), or Greiner Bio-One, Inc. ((www.) greinerbiooneinc.com). For example, silanated and silyated slides with free amino and aldehyde groups, respectively, are available and permit covalent coupling of molecules (e.g., polynucleotides with free aldehyde, amine, or other reactive groups) to the slides. As another example, slides with surface streptavidin are available and can bind biotinylated capture probes. In addition, services that produce arrays of polynucleotides of the customer's choice are commercially available, e.g., from TeleChem International ((www.) arrayit.com) and Agilent Technologies (Palo Alto, CA).

[0163] Suitable instruments, software, and the like for analyzing arrays to distinguish selected positions on the solid support and to detect the presence or absence of a label (e.g., a fluorescently labeled label probe) at each position are commercially available. For example, microarray readers are available, e.g., from Agilent Technologies (Palo Alto, CA), Affymetrix (Santa Clara, CA), and Zeptosens (Switzerland).

EXAMPLES

[0164] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art.
Accordingly, the following examples are offered to illustrate, but not to limit, the claimed invention.

EXAMPLE 1: A MULTIPLEX BRANCHED DNA ASSAY FOR PARALLEL QUANTITATIVE GENE EXPRESSION PROFILING

[0165] The following sets forth a series of experiments that demonstrate detection of nucleic acids using a multiplex bDNA assay, in which the nucleic acids are captured to distinct subsets of microspheres through cooperative hybridization. This example describes a new method to quantitatively measure the expression of multiple mRNAs directly from crude cell lysates and tissue homogenates without the need for RNA purification or target amplification. A distinguishing feature of this multiplex bDNA assay is the use of cooperative hybridization (a strong, stable hybridization interaction formed by the collective force of multiple weak, unstable hybridization interactions) in the assay design, which ensures an exceptionally high degree of assay specificity. The experiments demonstrate that the assay is capable of detecting a single transcript of mRNA per cell from as few as 25,000 cells and with intra-plate and inter-plate coefficients of variance (CV) of less than 10% and 15%, respectively. To demonstrate the utility of this technology, several 10-plex (ten-gene) panels were developed to quantify the expression of genes involved in the proinflammatory and apoptosis stimulation pathways. The data from the multiplex bDNA assay was compared with that of single-plex bDNA assays with a high degree of correlation. The multiplex bDNA assay thus provides a powerful means to quantify the gene expression profile of a defined set of target genes in large sample populations.

[0166] As described above, the branched DNA (bDNA) assay provides a useful approach for the quantification of mRNA transcripts. A significant departure from target amplification methods, the bDNA assay can directly measure mRNA from crude cell lysates and tissue homogenates by amplifying the reporter signal, and thus avoiding the errors inherent in the extraction and amplification of target sequences. Owing to its robustness and precision, the bDNA assay has been used in clinical diagnostic applications such as viral load determination, and in drug discovery research studying drug metabolism, structure-activity relationships, high throughput screens, and more recently, siRNA knockdown analysis (Urdea et al. (1991) "Branched DNA amplification multimers for the sensitive, direct detection of human hepatitis virus" Nuc Acids Symp Ser 24:197-200, Gleaves et al. (2002) "Multicenter evaluation of the Bayer VERSANT HIV-1 RNA 3.0 assay: analytical and clinical performance" J Clin Virol. 25:205-16, Hartley and Klaassen (2000) "Detection of chemical-induced differential expression of rat hepatic cytochrome P450 mRNA transcripts using branched DNA signal amplification technology" Drug Metab Dispos. 28:608-16, Bramlett et al. (2000) "Correlation of farnesoid X receptor coactivator recruitment and cholesterol 7alpha-hydroxylase gene repression by bile acids" Mol Genet Metab. 71:609-15, and Warrior et al. (2000) "Application of QuantiGene nucleic acid quantification technology for high throughput screening" J Biomol Screen. 5:343-52, and Soutschek et al. (2004) "Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs" Nature 432:173-8).

[0167] The Luminex bead-based array system has been used in a wide range of multiplex applications throughout the drug-discovery and diagnostics fields, as well as basic research (Iannone (2000) "Multiplexed single nucleotide polymorphism genotyping by oligonucleotide ligation and flow cytometry" Cytometry 39:131-40, Yang et al. (2001) "BADGE, Beads Array for the Detection of Gene Expression, a high-throughput diagnostic bioassay" Genome Res. 11: 1888-98, and Fulton et al. (1997) "Advanced multiplexed analysis with the FlowMetrix system" Clin Chem. 43:1749-56). For example, it has been a widely adopted platform for quantitative multiplex protein expression analysis. At the core are 100 fluorescent-encoded microsphere beads that can be coupled with a capture reagent specific to a particular bioassay, allowing for the detection of up to 100 unique analytes within a single sample.

[0168] A multiplex bDNA assay has been developed that combines the advantages of the conventional single-plex bDNA assay with, e.g., the multiplex capability of the Luminex platform. The fluorescent beads are coupled with a set of oligonucleotide capture probes, and cooperative hybridization is exploited in the assay design for exceptionally high assay specificity. The new mRNA quantification method measures the expression levels of multiple mRNA transcripts quantitatively from purified RNA and crude cell lysates with high accuracy and reproducibility. The simplicity and data quality of the assay makes it an ideal tool for high throughput parallel quantitative gene expression analysis.

Results

Overview of the multiplex bDNA assay

[0169] In the multiplex bDNA assay, fluorescent-encoded microsphere beads (Luminex Corporation) were used for the capture of specific mRNA species (**Figure 5 Panels A-C**). The ability to quantify multiple mRNA transcripts lies in the design of a probe set for each mRNA transcript of interest. A probe set contains three types of synthetic oligonucleotide probes called capture extenders (CEs, **501-503**), label extenders (LEs, **506-508**), and blockers (BPs, **510**) that hybridize and span a contiguous region of the target mRNA (**511-513**, provided for example in a cell lysate or tissue homogenate). The intended target is captured by multiple CEs, which hybridize specifically to their complementary capture probes (CPs, **521-523**) bound to the fluorescent-encoded beads (**526-528**) and allow the target mRNA to be captured to its corresponding beads. Signal amplification occurs when the LE tails are hybridized with a branched DNA molecule (bDNA amplifier, **530),** which can then be hybridized with biotinylated label probes **(531)** followed by streptavidin-conjugated phycoerythrin (SAPE, **532).** Bead fluorescent color codes and SAPE reporter signals from all beads in the final hybridization mixture are determined using a Luminex flow cytometer (or similar instrument), which maps each bead to a specific mRNA assay and provides a fluorescence measurement of SAPE reporter associated with that bead. The SAPE signals are proportional to the number of mRNA transcripts captured to the beads.

[0170] The assay includes three major steps: (1) Capture of the specific mRNA transcripts to their corresponding beads through CE-CP interaction during an overnight hybridization at 53°C **(Figure 5 Panel A);** (2) Hybridization with the bDNA amplification molecule and biotinylated label probe, respectively, for an hour at 46°C **(Figure 5 Panel B);** and (3) Hybridization with streptavidin-conjugated R-phycoerythrin (SAPE) at room temperature for 30 minutes **(Figure 5 Panel C).** The Luminex beads are then analyzed with a Luminex 100™ system. The level of SAPE fluorescence measured from each bead is proportional to the amount of mRNA transcript captured by the bead.

Cooperative hybridization

[0171] When the melting temperature (T_m) of an oligonucleotide duplex (e.g., a CP-CE hybridization interaction) is below the actual hybridization temperature, dissociation to its single-stranded form is favored under the assay condition. Such single hybridization does not result in stable capture of the target mRNA under this condition. However, when multiple weak hybridization interactions exist, the collective force of these weak hybridization allows the stable capture of the target mRNA. **Figure 6 Panels A** and **B** depict simple and cooperative hybridization, respectively. Simple hybridization between a CP and CE under the standard bDNA assay conditions is illustrated by the results shown in **Figure 6 Panel C.** CP14, CP15, and CP16 represent capture probes where the complementary sequence between CP and CE are 14, 15, or 16 bases in length, respectively. CP-N represents a nonspecific capture probe that does not hybridize with the CE sequence. The CE-LE represents a probe that can bind to the CP at one end and the bDNA amplification molecule at the other end. The CE represents a probe that binds to CP only but not the bDNA amplification molecule. Cooperative hybridization between CP and CE under the standard bDNA assay conditions is illustrated by the results shown in **Figure 6 Panel D.** A standard single-plex bDNA assay on IL-6 IVT RNA dilution series was performed in assay plate wells where CP14, CP15, or CP16 was attached to the surface, respectively. RLU stands for the Relative Luminescent Unit and the error bars represent 1 s.d.

[0172] A strong, stable hybridization interaction can be produced through the joint force of multiple weak, unstable hybridization interactions. The weak, unstable hybridization interaction occurs when the melting temperature (T_m) of the hybridizing nucleic acids is below the assay temperature. This concept is termed cooperative hybridization. Cooperative hybridization can be applied in bDNA assays to allow highly specific capture of target mRNA. When simple hybridization

(**Figure 6 Panel A**) between the CE and the CP occurs at a temperature above their melting temperature, the target mRNA cannot be stably captured to the solid surface. However, when multiple of those weak CE-CP hybridizations occur at the same time (**Figure 6 Panel B**), the target mRNA can be stably captured to the solid surface. To demonstrate cooperative hybridization, three capture probes varying in length from 14mer to 16mer were designed and used in a plate-based, single-plex bDNA assay. The 14 to 16 base capture probes were chosen because the melting temperature (T_m) is below the hybridization temperature of the bDNA assay, which is at 53°C. The simple hybridization between the CP and the CE was evaluated through an oligonucleotide (CE-LE) that contains a CE tail sequence complementary to the capture probe and an LE tail sequence complementary to a sequence in the bDNA amplifier molecule. A strong assay signal is obtained when the capture probe is 16 bases in length, a 100-fold weaker hybridization interaction is evident when the capture probe is 15 bases in length, and minimal hybridization interaction occurs when the capture probe is 14 bases in length (**Figure 6 Panel C**). When the three capture probes were used respectively in a bDNA assay where multiple CEs were present, overlapping assay signal was obtained regardless of capture probe length (**Figure 6 Panel D**). This suggests that cooperative hybridization plays a prominent role in the bDNA assay, especially when the capture probe is 14 bases long.

[0173] Using the cooperative hybridization concept, a set of capture probes that work together in a single hybridization reaction with minimal nonspecific cross-hybridization was designed. The capture probes were designed to comprise unique 15mer DNA sequences and a common linker that allows them to be chemically cross-linked onto the surface of the different fluorescent-encoded bead populations. Each bead population was examined for specific hybridization to its complementary biotinylated oligonucleotide as well as for its non-specific hybridization to the other biotinylated oligonucleotides. The assay signals of all possible non-specific hybridizations were less than 0.1 % of those observed for the perfectly matched pair, indicating a very high degree of hybridization specificity. Thus the set of capture probes was shown to be highly specific and can be used in the multiplex bDNA assay for the capture of target mRNA.

Performance evaluation of multiplex bDNA assay

[0174] To demonstrate the performance of the multiplex bDNA assay, several 10-plex panels were developed (three of which are described in **Table 1**) and evaluated for sensitivity, linear dynamic range, precision and specificity using in vitro transcribed (IVT) RNA transcripts as reference standards. The performance evaluation data for the cytokine panel (**Figure 7** and **Table 2**) is below. Probe sets for the cytokine panel are listed in **Table 3.**

[0175] To determine the cross-reactivity between target mRNAs, 40 amol of each IVT RNA transcript was individually hybridized into the bead array followed by measurement of signals for the intended target as well as for other genes. Cross-reactivity is expressed as the percentage of the average signal for other genes over the signal for the intended target. When 40 amol of individual mRNA transcripts (represented by gene name on the x-axis of **Figure 7 Panel A**) was added into the fluorescent-encoded bead array, only the corresponding bead (represented by number on the y-axis) gives a strong fluorescent signal (z-axis). Net MFI stands for background subtracted median fluorescent intensity from 100 counted beads. The cross-reactivity between target genes in the panel was less than 0.1%, suggesting minimal cross hybridization between the genes in the panel (**Figure 7 Panel A and Table 2**).

[0176] The assay specificity was evaluated by measuring the signal of *B. subtilis* dapB IVT RNA, which has little homology with human RNA, and human IL10 IVT RNA, which has undetectable expression in human U937 cells, as model genes. The IVT RNA transcripts of dapB and IL10 were serially diluted four-fold from 160 to 0.04 amol and mixed with 0.2 μg of total RNA extracted from human U937 cells. A multiplex bDNA assay was performed and the assay signals for dapB (**Figure 7 Panel B**) and IL10 (**Figure 7 Panel C**) were compared in the presence (squares) or absence (diamonds) of the U937 total RNA background. Overlapping signals were observed through the entire dilution series for both dapB and IL10, suggesting that minimal nonspecific hybridization occurred. Importantly, the addition of 0.2 μg of total RNA did not increase the assay background, further demonstrating the assay specificity. Tests that determine spike recovery of a target from a complex mixture are a typically recommended analytical procedure to assess assay accuracy. The assay is considered reliable if the signal difference between pure and spiked-in analyte is within ±20%. In this case, no change in assay signal was observed in the presence of U937 total RNA, indicating that nonspecific RNA does not seem to interfere with the hybridization of the target RNA to the capture beads. As a result, IVT RNA standard curves can be used to quantify the absolute number of copies of RNA transcripts in a sample.

[0177] To determine the assay sensitivity and linear dynamic range, ten target IVT RNA transcripts were equally mixed and serially diluted four-fold to generate standard curves with target RNA levels ranging from 2.4x 10^4 to 9.6x 16^7 transcripts (0.04 to 160 amol). The signal responses for all the cytokine genes were linear across the target concentration range examined with coefficients of correlation (R^2) averaged 0.99, indicating that the linear dynamic range of the assay spans more than 3 logs (**Figure 7 Panel D**). The sensitivity of the assay was evaluated by determining the limit of detection (LOD) for each target RNA. LOD, defined as the target concentration at which the signal is three standard deviations above the background, is 0.06 amol for VEGF and 0.04 amol for the remaining cytokines (**Table 2**).

[0178] Assay precision between different wells (intra-plate) and between assays performed on different days (inter-plate) was assessed by calculating coefficients of variation (%CV) for each gene expression measurement from 0.04 -160 amol IVT across multiple samples. Precision value for each gene was measured across multiple wells within a single plate (n = 4) and across multiple plates (N = 3). Intra-plate CV averaged about 8%, ranging from 5 to 14%, and inter-plate CV averaged about 14%, ranging from 7% to 22% (**Table 2**). Average intra-plate CV of less than 10% has been routinely obtained for multiplex bDNA assays with triplicate samples. It should be noted that the CVs were highly comparable from high to low concentrations of the target IVT RNA tested, suggesting that accurate quantification can be achieved in a target concentration-independent fashion.

[0179]

Table 1. Target names and reference sequence accession numbers for the cytokine and apoptosis 10-plex panels.

	Cytokine Panel		Apoptosis Panel 1		Apoptosis Panel 2	
Bead Number	Target Symbol	Accession Number	Target Symbol	Accession Number	Target Symbol	Accession. Number
22	IL2	NM_000586	RELB	NM_006509	BAK1	NM_001188
29	TNF	NM_000594	TNF	NM_000594	TNFSF6	NM_000639
19	VEGF	NM_003376	NFKB2	NM_002502	IL6R	NM_000565
28	IL10	NM_000572	TNFAIP3	NM_006290	TNFRSF6	NM_000043
25	IL6	NM_000600	IL6	NM_000600	PTK2B	NM_004103
33	IL1B	NM_000576	CDKN1A	NM_000389	BAD	NM_004322
20	IFNG	NM_000619	NFKB1	NM_003998	BCL2	NM_000633
18	IL8	NM_000584	RELA	NM_021975	BCL2L1	NM_138578
27	CSF2	NM_000758	NFKBIA	NM_020529	CFLAR	NM_003879
26	GAPD	NM_002046	GAPD	NM_002046	ACTB	NM_001101
34	dapB	L38424	N/A	N/A	N/A	N/A

[0180]

Table 2. Detection sensitivity, cross-reactivity, and assay background of the cytokine 10-plex panel.

Gene	CSF2	GAPD	IFNG	IL10	IL1B	IL2	IL6	IL8	TNF	VEG F	dapB
LOD (amol)	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.06	0.04
% Cross-reactivity*	0.004 %	0.003 %	0.006 %	0.007 %	0.004 %	0.006 %	0.018 %	0.100 %	0.002 %	0.139 %	0.018 %
Average background(MFI)	2.4	2.3	1.9	3.6	3.7	1.9	2.0	1.1	3.0	15.9	5.5
% Intra-plate CV	9%	8%	8%	7%	7%	5%	8%	14%	9%	7%	5%
% Inter-plate CV	21%	12%	11%	10%	22%	7%	13%	10%	18%	20%	7%
*Cross-reactivity is the percentage of signal generated by hybridization of mRNA to nontarget beads, in relation to the specific hybridization to its target bead (100%)											

[0181]

Table 3. Probe sets (CPs, CEs, LEs, and BPs) for the cytokine panel.

Gene			SEQ ID NO
CSF2	BP	GGGCTGGGCGAGCGG	5
CSF2	BP	CCAGGGCTGCGTGCTG	6
CSF2	BP	AGACGCCGGGCCTCC	7
CSF2	BP	CCGCAGGCCCTGCTTG	8
CSF2	BP	TCATGGTCAAGGGGCCCT	9
CSF2	BP	GGGGTTGGAGGGCAGTGC	10
CSF2	BP	CAGCAGTCAAAGGGGATGACA	11
CSF2	LE	caggccacagtgcccaagTTTTTaggcataggacccgtgtct	12
CSF2	LE	tggatggcattcacatgctcTTTTTaggcataggacccgtgtct	13
CSF2	LE	gcagtgtctctactcaggttcaggTTTTTaggcataggacccgtgtct	14
CSF2	LE	ttctactgtttcattcatctcagcaTTTTTaggcataggacccgtgtct	15
CSF2	LE	ggaggtcaaacatttctgagatgacTTTTTaggcataggacccgtgtct	16
CSF2	LE	tgtaggcaggtcggctcctTTTTTaggcataggacccgtgtct	17
CSF2	LE	gggttgcacaggaagtttccTTTTTaggcataggacccgtgtct	18
CSF2	LE	tttgaaactttcaaaggtgataatctTTTTTaggcataggacccgtgtct	19
CSF2	LE	agcagaaagtccttcaggttctcTTTTTaggcataggacccgtgtct	20
CSF2	LE	ctcactcctggactggctccTTTTTaggcataggacccgtgtct	21
CSF2	CE	agcagcaggctctgcagcTTTTTttgtgcagtgttata	22
CSF2	CE	gcgggtgcagagatgctgTTTTTttgtgcagtgttata	23
CSF2	CE	tacagctccaggcgggtcTTTTTttgtgcagtgttata	24
CSF2	CE	tgagcttggtgaggctgccTTTTTttgtgcagtgttata	25
CSF2	CE	tgcttgtagtggctggccaTTTTTttgtgcagtgttata	26
CSF2	CP	TTTTTTTTTATAACACTGCACAA	27
GAPD	BP	TCAGCGCCAGCATCGC	28
GAPD	BP	GAGGGGGCAGAGATGATGAC	29
GAPD	BP	AACATGGGGGCATCAGCA	30
GAPD	BP	CATGGTTCACACCCATGACG	31
GAPD	BP	CGGAGGGGCCATCCAC	32
GAPD	BP	TGGAGAGCCCCGCGG	33
GAPD	BP	gcaggaggcattgctgatga	34
GAPD	LE	gatgggatttccattgatgacaTTTTTaggcataggacccgtgtct	35
GAPD	LE	cccacttgattttggagggaTTTTTaggcataggacccgtgtct	36
GAPD	LE	ccagtggactccacgacgtacTTTTTaggcataggacccgtgtct	37
GAPD	LE	ttctccatggtggtgaagacgTTTTTaggcataggacccgtgtct	38
GAPD	LE	tcttgaggctgttgtcatacttctTTTTTaggcataggacccgtgtct	39
GAPD	LE	gggtgctaagcagttggtggtTTTTTaggcataggacccgtgtct	40
GAPD	LE	ccttccacgataccaaagttgtTTTTTaggcataggacccgtgtct	41

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Gene			SEQ ID NO
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GAPD	LE	agtcttctgggtggcagtgatTTTTTaggcataggacccgtgtct	43
GAPD	LE	ccatcacgccacagtttccTTTTTaggcataggacccgtgtct	44
GAPD	LE	cagtagaggcagggatgatgttcTTTTTaggcataggacccgtgtct	45
GAPD	LE	cacagccttggcagcgcTTTTTaggcataggacccgtgtct	46
GAPD	LE	ccagtgagcttcccgttcaTTTTTaggcataggacccgtgtct	47
GAPD	CE	tgacggtgccatggaatttTTTTTaaaactatacgtgct	48
GAPD	CE	agcttcccgttctcagcctTTTTTaaaactatacgtgct	49
GAPD	CE	tctcgctcctggaagatggtTTTTTaaaactatacgtgct	50
GAPD	CE	gcaaatgagccccagccTTTTTaaaactatacgtgct	51
GAPD	CE	ccttttggctccccccTTTTTaaaactatacgtgct .	52
GAPD	CE	catggatgaccttggccagTTTTTaaaactatacgtgct	53
GAPD	CE	gctcagggatgaccttgccTTTTTaaaactatacgtgct	54
GAPD	CP	TTTTTTTTAGCACGTATAGTTTT	55
IFNG	BP	TGCATTAAAATATTTCTTAAGGTTTTCT	56
IFNG	BP	AAATGCCTAAGAAAAGAGTTCCA	57
IFNG	BP	AAAAAGTTTGAAGTAAAAGGAGACAAT	58
IFNG	BP	GATGCTCTGGTCATCTTTAAAGTTTTT	59
IFNG	BP	GGATGCTCTTCGACCTTGAAAC	60
IFNG	BP	AATAAATAGATTTAGATTTAAAATTCAAATATT	61
IFNG	LE	gcttcttttacatatgggtcctggTTTTTaggcataggacccgtgtct	62
IFNG	LE	ttatccgctacatctgaatgaccTTTTTaggcataggacccgtgtct	63
IFNG	LE	ttgatggtctccacactcttttgTTTTTaggcataggacccgtgtct	64
IFNG	LE	aaaaacttgacattcatgtcttccTTTTTaggcataggacccgtgtct	65
IFNG	LE	ataattagtcagcttttcgaagtcaTTTTTaggcataggacccgtgtct	66
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IFNG	LE	gcaggcaggacaaccattactgTTTTTaggcataggacccgtgtct	70
IFNG	LE	aatacttatttgattgatgagtctaaaaatTTTTTaggcataggacccgtgtct	71
IFNG	CE	cactctcctctttccaattcttcaTTTTTTTTttcacacacattaac	72
IFNG	CE	ttggctctgcattatttttctgtTTTTTttcacacacattaac	73
IFNG	CE	tctcgtttctttttgttgctattgTTTTTttcacacacattaac	74
IFNG	CE	atgagttcatgtattgctttgcgtTTTTTttcacacacattaac	75
IFNG	CE	ttccctgttttagctgctggTTTTTttcacacacattaac	76
IFNG	CE	atattccccatataaataatgttaaatattTTTTTttcacacacattaac	77
IFNG	CP	TTTTTTTTGTTAATGTGTGTGAA	78

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Gene			SEQ ID NO
IL1	BP	GGTAAAACTGGATCATCTCAGACAA	79
IL1	BP	GGGGCATCACCTCCTCCA	80
IL1	BP	GTTCACATGCGCCTTGATGT	81
IL1	BP	GCTCTTGTTTTCACAGGGAAGA	82
IL1	BP	GGCTTTGTAGATGCCTTTCTCT	83
IL1	BP	gactgggtgccctggcc	84
IL1	LE	TaggcaggttgcctgggaTTTTTaggcataggacccgtgtct	85
IL1	LE	gtcttcactctgctgaaggcatTTTTTaggcataggacccgtgtct	86
IL1	LE	actcctttaacaacaagttgtccaTTTTTaggcataggacccgtgtct	87
IL1	LE	ccttaaagtcctccagcaaggTTTTTaggcataggacccgtgtct	88
IL1	LE	ggcttggcaacccaggtaacTTTTTaggcataggacccgtgtct	89
IL1	LE	caggttctcccccagggaTTTTTaggcataggacccgtgtct	90
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IL1	CE	ctgggtcttggttctcagcttTTTTTccgtgcttttctaat	102
IL1	CE	gcctcagcctgagggtcttTTTTTccgtgcttttctaat	103
IL1	CE	ccgattttggagacctctaatttaTTTTTccgtgcttttctaat	104
IL1	CP	TTTTTTTTATTAGAAAAGCACGG	105
IL1B	BP	ACTGACGCGGCCTGCC	106
IL1B	BP	ccagacatcaccaagctttttt	107
IL1B	LE	gccatcagcttcaaagaacaagTTTTTaggcataggacccgtgtct	108
IL1B	LE	aaggagcacttcatctgtttaggTTTTTaggcataggacccgtgtct	109
IL1B	LE	atgccgccatccagaggTTTTTaggcataggacccgtgtct	110
IL1B	LE	ggtcggagattcgtagctggTTTTTaggcataggacccgtgtct	111
IL1B	LE	gcttgtccatggccacaacaTTTTTaggcataggacccgtgtct	112
IL1B	LE	gggaaccagcatcttcctcaTTTTTaggcataggacccgtgtct	113
IL1B	LE	ggttcttcttcaaagatgaagggTTTTTaggcataggacccgtgtct	114
IL1B	LE	ttatcccatgtgtcgaagaagataTTTTTaggcataggacccgtgtct	115

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Gene			SEQ ID NO
IL1B	LE	catcgtgcacataagcctcgTTTTTaggcataggacccgtgtct	116
IL1B	LE	gcagttcagtgatcgtacaggtgTTTTTaggcataggacccgtgtct	117
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IL1B	LE	aggccacaggtattttgtcattTTTTTaggcataggacccgtgtct	121
IL1B	CE	gcagaggtccaggtcctggTTTTTaacgtgtattccatt	122
IL1B	CE	tgaagcccttgctgtagtggtTTTTTaacgtgtattccatt	123
IL1B	CE	cctggaaggtctgtgggcaTTTTTaacgtgtattccatt	124
IL1B	CE	aaagaaggtgctcaggtcattctTTTTTaacgtgtattccatt	125
IL1B	CE	ggagagctttcagttcatatggaTTTTTaacgtgtattccatt	126
IL1B	CE	ccatatcctgtccctggaggtTTTTTaacgtgtattccatt	127
IL1B	CE	attcttttccttgaggcccaTTTTTaacgtgtattccatt	128
IL1B	CP	TTTTTTTTAATGGAATACACGTT	129
IL2	BP	TTCCATTCAAAATCATCTGTAAATC	130
IL2	BP	CCTGGGTCTTAAGTGAAAGTTTTT	131
IL2	BP	AGCATATTCACACATGAATGTTGTT	132
IL2	BP	AAAAGGTAATCCATCTGTTCAGAAA	133
IL2	BP	ATTCAACAATAAATATAAAATTTAAATATfiTA	134
IL2	LE	agtaggtgcactgtttgtgacaagTTTTTaggcataggacccgtgtct	135
IL2	LE	gctgtgttttctttgtagaacttgaTTTTTaggcataggacccgtgtct	136
IL2	LE	cagcagtaaatgctccagttgtaTTTTTaggcataggacccgtgtct	137
IL2	LE	aaacttaaatgtgagcatcctggTTTTTaggcataggacccgtgtct	138
IL2	LE	tagacactgaagatgtttcagttctgTTTTTaggcataggacccgtgtct	139
IL2	LE	gctttgagctaaatttagcacttcTTTTTaggcataggacccgtgtct	140
IL2	LE	attacgttgatattgctgattaagtcTTTTTaggcataggacccgtgtct	141
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IL2	CE	tgagtttgggattcttgtaattattaaTTTTTgaagttaccgttttc	146
IL2	CE	tggccttcttgggcatgtaTTTTTgaagttaccgttttc	147
IL2	CE	ctccagaggtttgagttcttcttcTTTTTgaagttaccgttttc	148
IL2	CE	tcagatccctttagttccagaactTTTTTgaagttaccgttttc	149
IL2	CE	aataaatagaaggcctgatatgttttaTTTTTgaagttaccgttttc	150
IL2	CP	TTTTTTTTGAAAACGGTAACTTC	151
IL6	BP	TGGGGCAGGGAAGGCA	152

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Gene			SEQ ID NO
IL6	BP	GGAATCTTCTCCTGGGGGTAC	153
IL6	BP	TGGGGCGGCTACATCTTT	154
IL6	BP	GCTTTCACACATGTTACTCTTGTTACA	155
IL6	BP	TTTGGAAGGTTCAGGTTGTTTT	156
IL6	BP	CCTCAAACTCCAAAAGACCAGTG	157
IL6	BP	TTGGGTCAGGGGTGGTTATT	158
IL6	BP	CTGCAGGAACTCCTTAAAGCTG	159
IL6	BP	CCCATTAACAACAACAATCTGAGG	160
IL26	LE	ggctcctggaggcgagataTTTTTaggcataggacccgtgtct	161
IL6	LE	aactggaccgaaggcgctTTTTTaggcataggacccgtgtct	162
IL6	LE	gcaggcaacaccaggagcTTTTTaggcataggacccgtgtct	163
IL6	LE	aagaggtgagtggctgtctgtgTTTTTaggcataggacccgtgtct	164
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IL6	LE	gcatccatctttttcagccatcTTTTTaggcataggacccgtgtct	168
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IL6	CE	cctcattgaatccagattggaaTTTTTggggaacatagaaaa	186
IL6	CE	gaagagccctcaggctggaTTTTTggggaacatagaaaa	187
IL6	CP	TTTTTTTTTTTTCTATGTTCCCC	188
IL8	BP	CAAAAACTTCTCCACAACCCTC	189

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Gene			SEQ ID NO
IL8	BP	AGTGTTGAAGTAGATTTGCTTGAAGT	190
IL8	BP	CAACAGACCCACACAATACATGA	191
IL8	BP	GTACAATGAAAAACTATTCATTGTTTACT	192
IL8	BP	TTTTTTGTAGATTCAAATAAATAATACTTTA	193
IL8	BP	AAATCCTTATATTTAAAAATTATTTGTTG	194
IL8	BP	GCTTCAAATATCACATTCTAGCAAAC	195
IL8	BP	AAAAAATCCAGGATTTCCAGCt	196
IL8	BP	CTAGGGTTGCCAGATTTAACAGA	197
IL8	BP	CCACTTAGAAATAAAGGAGAAACCA	198
IL8	BP	CATGTCCTCACAACATCACTGTGA	199
IL8	BP	ATGAAAAAACTTAAAGTGCTTCCA	200
IL8	BP	AAGTTACACTTGAAAATAATTTATGTTATG	201
IL8	BP	TTAAATAAATACATAAATAATAA.ATAGGTTAAT	202
IL8	BP	ATAAAACATCATTTAATATCTAAAATAAAAT	203
IL8	BP	TAAAAACCCTGATTGAAATTTATCTA	204
IL8	LE	ggtccagacagagctctcttccTTTTTaggcataggacccgtgtct	205
IL8	LE	ttggataccacagagaatgaatttTTTTTaggcataggacccgtgtct	206
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IL8	LE	aattcttgcacaaatatttgatgcTTTTTaggcataggacccgtgtct	218
IL8	LE	caatgattcatcttctatttttccaTTTTTaggcataggacccgtgtct	219
IL8	LE	aaatttactataacatctttataactattcaatTTTTTaggcataggacccgtgtct	220
IL8	CE	tgcacccagttttccttggTTTTTttcaaatgttagcct	221
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IL8	CE	cggatattctcttggcccttTTTTTttcaaatgttagcct	223
IL8	CE	tgtggatcctggctagcagaTTTTTttcaaatgttagcct	224
IL8	CE	acccaattgtttgtttgtttaatcTTTTTttcaaatgttagcct	225

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Gene			SEQ ID NO
IL8	CP	TTTTTTTTAGGCTAACATTTGAA	226
TNF	BP	CCCTCTGGGGGCCGA	227
TNF	BP	GAGGTCCCTGGGGAACTCTT	228
TNF	BP	ggccagagggctgattagaga	229
TNF	BP	AGGCTTGTCACTCGGGGTT	230
TNF	BP	tgaagaggacctgggagtagatg	231
TNF	BP	GGGCAGCCTTGGCCCT	232
TNF	BP	TGGCAGGGGCTCTTGATG	233
TNF	BP	CCCCTCTGGGGTCTCCCTC	234
TNF	BP	GTTTGGGAAGGTTGGATGTTC	235
TNF	BP	TGGGGCAGGGGAGGC	236
TNF	BP	AGGAGGGGGTAATAAAGGGAT	237
TNF	LE	tcactccaaagtgcagcaggTTTTTaggcataggacccgtgtct	238
TNF	LE	ggtttgctacaacatgggctacTTTTTaggcataggacccgtgtct	239
TNF	LE	ggcggttcagccactggaTTTTTaggcataggacccgtgtct	240
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TNF	LE	tgaggagcacatgggtggagTTTTTaggcataggacccgtgtct	245
TNF	LE	gcggctgatggtgtgggTTTTTaggcataggacccgtgtct	246
TNF	LE	gcagagaggaggttgaccttgTTTTTaggcataggacccgtgtct	247
TNF	LE	cagggcttggcctcagcTTTTTaggcataggacccgtgtct	248
TNF	LE	tctccagctggaagaccccTTTTTaggcataggacccgtgtct	249
TNF	LE	gcgctgagtcggtcaccctTTTTTaggcataggacccgtgtct	250
TNF	LE	agactcggcaaagtcgagatagTTTTTaggcataggacccgtgtct	251
TNF	LE	atcccaaagtagacctgcccTTTTTaggcataggacccgtgtct	252
TNF	LE	gtcctcctcacagggcaatgTTTTTaggcataggacccgtgtct	253
TNF	LE	cagaagaggttgagggtgtctgaTTTTTaggcataggacccgtgtct	254
TNF	LE	gcttgggttccgaccctaagTTTTTaggcataggacccgtgtct	255
TNF	CE	cgagaagatgatctgactgcctgTTTTTctgagtcaaagcatt	256
TNF	CE	gctgcccctcagcttgagTTTTTctgagtcaaagcatt	257
TNF	CE	gtctggtaggagacggcgatTTTTTctgagtcaaagcatt	258
TNF	CE	tcccagatagatgggctcatacTTTTTctgagtcaaagcatt	259
TNF	CE	tcgggccgattgatctcaTTTTTctgagtcaaagcatt	260
TNF	CE	cccccaattctcttttgagcTTTTTctgagtcaaagcatt	261
TNF	CP	TTTTTTTTAATGCTTTGACTCAG	262

(continued)

Gene			SEQ ID NO
VEGF	BP	CATCAGGGGCACACAGGATG	263
VEGF	BP	GCAGCCCCCGCATCG	264
VEGF	BP	CTCCTCAGTGGGCACACACTC	265
VEGF	LE	tggaggtagagcagcaaggcTTTTTaggcataggacccgtgtct	266
VEGF	LE	tgggaccacttggcatggTTTTTaggcataggacccgtgtct	267
VEGF	LE	gatgattctgccctcctccttTTTTTaggcataggacccgtgtct	268
VEGF	LE	tccatgaacttcaccacttcgtTTTTTaggcataggacccgtgtct	269
VEGF	LE	gcagtagctgcgctgatagacaTTTTTaggcataggacccgtgtct	270
VEGF	LE	accagggtctcgattggatgTTTTTaggcataggacccgtgtct	271
VEGF	LE	agggtactcctggaagatgtccTTTTTaggcataggacccgtgtct	272
VEGF	LE	gcttgaagatgtactcgatctcatcTTTTTaggcataggacccgtgtct	273
VEGF	LE	caggccctcgtcattgcaTTTTTaggcataggacccgtgtct	274
VEGF	LE	TaatctgcatggtgatgttggaTTTTTaggcataggacccgtgtct	275
VEGF	LE	atttgttgtgctgtaggaagctcTTTTTaggcataggacccgtgtct	276
VEGF	LE	ctgattttttttcttgtcttgctctTTTTTaggcataggacccgtgtct	277
VEGF	LE	ttgcgctttcgtttttgcTTTTTaggcataggacccgtgtct	278
VEGF	LE	ggcccacagggaacgctTTTTTaggcataggacccgtgtct	279
VEGF	CE	aaggctccaatgcacccaTTTTTctttgagttcggttt	280
VEGF	CE	ctgccatgggtgcagccTTTTTctttgagttcggttt	281
VEGF	CE	tggtgaggtttgatccgcaTTTTTctttgagttcggttt	282
VEGF	CE	atctctcctatgtgctggcctTTTTTctttgagttcggttt	283
VEGF	CE	atctttctttggtctgcattcacTTTTTctttgagttcggttt	284
VEGF	CE	ccctttccctttcctcgaaTTTTTctttgagttcggttt	285
VEGF	CE	ccaggacttataccgggatttcTTTTTctttgagttcggttt	286
VEGF	CP	TTTTTTTTAAACCGAACTCAAAG	287

Regulation of gene expression from cellular models of inflammation and apoptosis

[0182] To demonstrate the utility of this assay using cell lysates, two well-characterized cell culture model systems that elicit expression changes in genes were employed within the panels. The first model system utilized PMA/LPS treatment of U937 cells to monitor the changes of cytokine gene expression in inflammatory responses, and the second model utilized TNFα-treated HeLa cells to monitor expression changes in pro- and anti-apoptotic genes.

[0183] U937 cells with or without PMA/LPS treatment were lysed in the culture flask by adding a lysis buffer (from QuantiGene and QuantiGene Plex kit) directly to the culture media. Since the lysis buffer facilitates the release and hybridization of intact mRNA, crude cell lysates were used directly for all multiplex bDNA assays. Gene expression was measured in the 10-plex cytokine panel for control and PMA/LPS treated U937 cells. 40,000 cell equivalents of cell lysate was used in the assay. When the assay signal for a gene was below the detection limit, the expression level of that gene was not plotted. The measurements were performed in quadruplicate and data acquired from Luminex 100™ is normalized to the housekeeping gene GAPD. Error bar represents 1 s.d.

[0184] As shown in **Figure 8 Panel A,** untreated U937 cells, GAPDH and VEGF were expressed at a high level, IL8, IL1b, and TNFα were expressed at moderate to low level, and IL6, IL10, CSF2 (GM-CSF), IL2, and IFN-γ are not significantly above the background. PMA/LPS treatment strongly induced the expression of proinflammatory cytokines IL8, IL-1b and TNFα. However, cytokines IL6, IL10 and CSF2 were only moderately induced, while IFN-γ and IL2 and

VEGF remain essentially unchanged. The average %CV or the measurements (including both signal and background) for uninduced cells was 12.8%, and for induced cells was 8.9%. The signals of the induced genes increased linearly with the increasing amount of cell lysate (e.g., 6,000 to 25,000 cells) assayed, while the background level remained the same throughout, demonstrating the specific nature of the assay signal. Further verification of mRNA levels from the same cell lysates was performed using the well-established single-plex bDNA assay (**Figure 8 Panel B**) . 20,000 cell equivalents of cell lysate was used in the assay. The measurements were performed in quadruplicate and data normalized to 40,000 cell equivalents and to housekeeping gene GAPD. Error bar represents 1 s.d. The results showed a highly similar pattern of gene expression to the results of the multiplex assay, with a correlation factor of 0.94 by matched pair analysis (**Figure 8 Panel B**).

[0185] To further demonstrate the utility of the multiplex assay, two 10-plex apoptosis panels were developed and used to analyze gene expression from HeLa cells treated with TNFα for 1 to 6 hours. One apoptosis panel measures the expression of several major subunits of the NF-kB transcription complexes, including RelA, RelB, NFKB1, NFKB2, and NFKB1A, as well as a number of additional pro- and anti-apoptotic regulators, and a number of cytokines (**Table 1**). Expression of individual genes in the 10-plex apoptosis panel with or without TNFα treatment for 0, 1, 3, or 6 hours is shown in **Figure 9 Panel A.** 80,000 cell equivalents of cell lysate was used in the assay except for GAPD where data was obtained from 10,000 cell equivalents of cell lysate. The measurements were performed in triplicate with data acquired from a Bio-Plex instrument at high sensitivity setting and normalized to housekeeping gene GAPD.

[0186] Several patterns of gene expression were observed (**Figure 9 Panel A**). The expression of A20 and TNFα was rapidly elevated after 1 hour, then declined after 3 hour, and further declined after 6 hour of TNFα stimulation. The expression of NFKB1, NFKB2, NFKB1A, and IL-6 was increased after 1 hour, and maintained at roughly the similar level (within 2-fold of each other) after 3 hour and 6 hour of TNFα stimulation. The expression of RELB increased moderately at 1 hour, but elevated at much higher level after 3 hours and 6 hours of TNFα stimulation. Consistent with their known functional role in the NF-kB transcription complex and in cell cycle control, the expression levels of RELA and CDKN1A did not change significantly upon TNFα treatment. The average %CV of all expression measurements was 8.5%. Verification tests of these results were performed by measuring the expression levels of all ten genes in control and 3 hour TNFα-treated samples with the single-plex bDNA assay. 40,000 cell equivalents of cell lysate was used in each assay except for GAPD where data was obtained from 10,000 cell equivalents of cell lysate. Measurements from both technologies reveal similar relative expression levels and fold-of-induction changes (**Figure 9 Panel B**). Correlation (R^2) between expression measurements for untreated samples was >0.99, and for treated samples R^2 >0.94 was observed across both panels.

[0187] Performance evaluation data for the two apoptosis panels is summarized in **Tables 4** and **5**.

[0188]

Table 4. Detection sensitivity, cross-reactivity, and assay background of the apoptosis 1 panel.

Gene	RELA	NFKB2	NKFB1	RELB	IL6	GAPD	NFKB1A	TNFAIP3	TNFA	CDKN1A
LOD (amol)	0.06	0.06	0.04	0.04	0.04	0.04	0.16	0.04	0.04	0.04
% Cross- reactivity	0.024 %	0.028 %	0.023 %	0.028%	0.005 %	0.02 %	0.017 %	0.019 %	0.044 %	0.023 %
Average background (MFI)	53.3	19.5	10.1	14.5	15.9	18.1	82.1	31.1	15.6	16
% Intra- plate CV	13	10	14	13	9	9	12	11	11	9
% Inter- plate CV	16	16	14	13	8	12	10	14	12	13

[0189]

Table 5. Detection sensitivity, cross-reactivity, and assay background of the apoptosis 2 panel.

Gene	BCL2L1	IL6R	BCL2	BAK1	PTK2B	ACTB	CFLAR	TNFRSF6	TNFSF6	BAD
LOD (amol)	0.04	0.04	0.16	0.16	0.04	0.04	0.06	0.04	0.04	0.16
% Cross-reactivity	0.021 %	0.005 %	0.038 %	0.072 %	0.006 %	0.016 %	0.021 %	0.038 %	0.345 %	0.024 %
Average background (MFI)	9.9	24.3	72.6	26.4	15.3	11.6	24.4	16.5	17.8	14.8
% Intra-plate CV	7	7	8	9	7	10	8	10	6	11
% Inter-plate CV	11	11	18	23	14	15	15	13	14	16

Advantages of the multiplex bDNA assays

[0190] The above experiments demonstrate that the multiplex bDNA assay is a simple and robust method for gene expression quantification that generates sensitive and reproducible results directly from crude cell lysates, without need for RNA purification, reverse transcription, or target amplifications. One distinctive feature of the assay is its remarkable specificity and accuracy. Cooperative hybridization was demonstrated in a single-plex bDNA assay. Cooperative hybridization can enable exceptionally high assay specificity, enabling multiplexing of the assay. A target mRNA transcript can only be stably captured to its corresponding beads when the transcript binds to multiple CEs. Even when an unintended mRNA transcript binds to one of the CEs in a probe set, the mRNA transcript cannot be stably captured to the beads. Thus, cooperative hybridization can potentially provide two major advantages over simple hybridization currently used in most hybridization based assays: 1) it dramatically reduces the assay background caused by nonspecific cross hybridization; and 2) it enables better discrimination of homologous genes. By leveraging cooperative hybridization in the design of the capture probes, less than 0.1% cross-reactivity has been achieved in all the multiplex panels developed so far, including a rat toxicity panel where several highly homologous cytochrome P450 genes are present. Specificity of the assay is further demonstrated by its excellent spike recovery in the presence of a complex background of total RNA. Because of its exceptional assay specificity, the results of mRNA quantification are therefore highly accurate and reliable.

[0191] Another distinctive feature of the assay is its simplicity. The assay can quantify RNA transcripts in crude cell lysates and tissue homogenates, as demonstrated in this study using cell lysates from two different cell lines. This feature distinguishes the assay from many other existing mRNA quantification technologies, and makes the assay one of the few that directly measures the mRNA transcripts from their native environment, thus free of any bias imposed by purification procedures and enzymatic reactions. By eliminating the RNA purification, reverse transcription, labeling and amplification steps, assay variations are minimized, leading to significantly improved precision for the overall assay. In addition, since the need for sample preparation is eliminated, the multiplex bDNA assay is well suited for high throughput expression analysis of large sample populations.

[0192] The third distinctive feature of the assay is its high precision and reproducibility. The intra-plate CV is routinely below 10% and the inter-plate CV routinely below 15% in all experiments where triplicate samples are run, whether the samples are total RNA or cell lysates. The level of consistency in multiplex bDNA assay data is a particularly attractive feature required for quantitative measurement of gene expression in siRNA knockdown, structure-activity relationship, drug dose response, and drug screening applications, where a change of gene expression as small as 10 to 30% needs to be reliably determined and differentiated.

[0193] Finally, the multiplex bDNA assay is capable of measuring multiple mRNA transcripts simultaneously with good sensitivity and broad dynamic range. The current assay can be expected to detect mRNA transcripts at a concentration as low as 1 copy per cell from a few as 25,000 cells (i.e., the detection sensitivity is 25,000 transcripts), and two mRNA transcripts differing in concentration by as much as 1,000-fold can both be quantified in the same assay with high accuracy. Sensitivity can optionally be increased by decreasing the assay volume (e.g., to 50 μl), including more LEs per probe set so that more amplification multimers bind to each mRNA, and/or using amplification multimers with a higher theoretical fold of amplification. Less than 0.1% cross-reactivity is routinely achieved in more than a dozen multiplex panels tested so far, including cytochrome P450 and ABC transporter panels where several genes with up to 96% homology are present. 30- and 40-plex bDNA assays have been performed, with the same performance characteristics.

[0194] Although the multiplex bDNA assays described herein simultaneously quantify ten mRNA targets in a single assay, the Luminex technology platform was designed for simultaneous measurement of up to 100 bead analytes. The number of targets in the multiplex bDNA assay can thus be readily increased, e.g., by taking advantage of this platform. Expansion of the multiplex bDNA assay will permit more detailed analysis of mRNA levels in particular pathways and enable more robust systems biology studies. Increased multiplex capability may be desirable for some applications such as microarray validation and follow up studies.

[0195] The multiplex bDNA assay enables collection of a large amount of data that previously were experimentally challenging to obtain. For example, a previously published study on the cytokine response to inflammatory stimuli was mostly focused on two to three cytokines at a time (Hass et al. (1991) "Regulation of TNF-alpha, IL-1 and IL-6 synthesis in differentiating human monoblastoid leukemic U937 cells" Leuk Res. 15:327-39, and Roberts et al. (1997) "Effects of Porphyromonas gingivalis and Escherichia coli lipopolysaccharides on mononuclear phagocytes" Infect Immun. 65: 3248-54). With the multiplex bDNA assay, the quantitative expression of dozens of cytokine genes can now be investigated. The multiplex expression analysis of time dependent regulation of apoptotic gene expression upon TNF-alpha treatment in HeLa cells illustrates the potential for high throughput quantitative gene expression analysis. The throughput of the multiplex bDNA assay enables the detailed expression analysis in many samples that received different drug treatments at various dose for different length of times.

[0196] In summary, the simplicity, sensitivity, accuracy and high reproducibility of the assay, together with the multiplex capability, makes the multiplex bDNA assay a valuable tool, e.g., in applications where accurate and robust quantification of multiple mRNA targets is required, where samples and reagents are precious and limited, and where easy and high throughput sample processing is desired, such as in biomarker validation, compound screening, structure-activity relationship study, toxicity studies, and potentially clinical diagnostics.

Methods

Cooperative hybridization

[0197] To determine the effect of cooperative hybridization, three oligonucleotide capture probes containing 14, 15, or 16 bases complementary to the CE tails were covalently conjugated to capture plates, respectively, following a published procedure (Running and Urdea (1990) "A procedure for productive coupling of synthetic oligonucleotides to polystyrene microtiter wells for hybridization capture" Biotechniques. 8:276-279). The sequences of the three capture probes are as follows: 16 base capture probe, 5'-amine-spacer-ACTTTCTTTCCAAGAG-3' (SEQ ID NO:1); 15 base capture probe, 5'-amine-spacer-ACTTTCTTTCCAAGA-3' (SEQ ID NO:2); and 14 base capture probe, 5'-amine-spacer-ACTTTCTTTCCAAG-3' (SEQ ID NO:3). Standard bDNA assays were run on the assay plates coupled with the three capture probes, respectively, using IVT RNA of IL-6 as standard and the bDNA assay probe set for IL-6. To determine the extent of simple hybridization, an oligonucleotide probe (CE-LE) that combines the sequences of the CE tail and the LE tail together was used in bDNA assay in place of the probe set and target RNA. The sequence of the CE-LE probe is as follow: 5'-AGGCATAGGACCCGTGTCtttttCTCTTGGAAAGAAAGT-3' (SEQ ID NO:4), where the series of five small letter t's denotes the linker sequence between the LE tail and the CE tail.

Single-plex bDNA assay

[0198] Single-plex bDNA assay was performed according to the Instruction Manual of the QuantiGene® Reagent System (Panomics). Briefly, cells were incubated with Lysis Mixture for 15 min at 37˚C in 100μL volume. Probe set for a target gene was added to the cell lysate, transferred to a capture well, and then incubated for 16 hr at 53˚C. Unbound material was washed from wells, followed by sequential hybridization at 46˚C with branched DNA (bDNA) amplifier and alkaline phosphatase-conjugated label probe. After a final wash, the alkaline phosphatase substrate dioxetane was added to wells and incubated at 46˚C for 30 min to develop luminescent signal, which was detected using an Lmax microtiter plate luminometer (Molecular Devices, Sunnyvale, CA).

Capture probes and their coupling to fluorescent-encoded beads

[0199] Unique sequences of 15 bases were chosen as capture probes. The capture probes were designed to have minimal potential for secondary structure formation or cross-hybridization. They were also screened against homology with sequences of human, mouse or rat genes (BLAST and NCBI databases) or sequences in the bDNA and label probe. Oligonucleotide capture probes were synthesized with 5'-amino linker (BioSearch) and covalently linked to carboxylated fluorescent-encoded microsphere beads (Luminex) following the recommended conjugation procedure from Luminex. Hybridization specificity was evaluated by hybridizing ten coupled microsphere beads to a biotinylated complementary oligonucleotide under the same hybridization conditions as the bDNA assay.

In vitro transcribed RNA (IVT RNA)

[0200] Complementary DNA clones encoding the full-length target genes were obtained commercially and used as templates for *in vitro* transcription to generate IVT RNA standards (Invitrogen; Origene; or Open Biosystems). IVT RNA was transcribed for each gene from the restriction enzyme digested plasmid via T3, T7, or SP6 promoters using the Ampliscribe kit (Amersham), quantified using RiboGreen fluorescence (Molecular Probes) and the Envision 2100 Multilabel reader (Perkin Elmer). IVT RNA transcripts for each panel were mixed and serially diluted four-fold to generate standard curve with target RNA levels ranging from 9.6 x 10^7 to 2.4 x 10^4 transcripts. The IVT RNA was used as a reference standard to assess the assay sensitivity, specificity, accuracy, and linear dynamic range.

Probe design for single-plex and multiplex bDNA assays

[0201] Probe design software (Bushnell et al. (1999) "ProbeDesigner: for the design of probe sets for branched DNA (bDNA) signal amplification assays Bioinformatics 15:348-sus) was modified to design probe sets for target genes in both single-plex and multiplex bDNA assays. For each target sequence, the software algorithm identifies regions that can serve as annealing templates for CEs (5-7 per gene), LEs (10-15 per gene), or BPs. Potential CEs and LEs were examined for possible interactions with other components of the multiplex assay, and CEs and LEs expected to cross-hybridize were not selected for use: CE-LE, CE-bDNA, CE-label probe, and LE-capture probe interactions having highly negative ΔG (e.g., < -7.0 kcal/mole) were removed to minimize non-specific hybridization. Probe sets are essentially the same for both single-plex and multiplex bDNA assay except for the portion of the CE probes that hybridize with capture probe. Several 10-plex panels were developed for the experimental validation. Gene names and reference sequence accession numbers are shown in **Table 1;** probe sets are shown in **Table 3.**

Multiplex bDNA assay

[0202] Multiplex bDNA assay was performed basically as described in the Instruction Manual of the QuantiGene® Plex Reagent System (Panomics). Briefly, samples containing IVT RNA, total RNA or cell lysates were mixed with the multiplex panel probe sets and the capture beads (about 2000 beads per subset) and hybridized for 16 hours at 53°C in 100μL volume. The components in a 100-μL IVT RNA assay were 33% lysis mixture, 40% capture buffer (Panomics), 1 μg tRNA, and the panel-specific probe set (CE, 0.165 fmol/μl/gene; LE, 0.66 fmol/μl/gene; BP, 0.33fmol/μl/gene). (Hybridization can be performed under any of a variety of suitable conditions; for example, in a solution including the capture diluent described in Collins ML et al. (1997) Nucleic Acid Research 25:2979-2984: 127 mM LiCl, 5% lithium lauroyl sulfate, 9 mM EDTA, 50 mM HEPES (pH 7.5), 0.05% hespan (DuPont Pharmaceuticals), 0.05% ProClin 300 (Supelco), and 0.2% casein (Research Organics, Hammarsten quality).) Hybridization reactions were transferred to a 0.45-μm filter plate (Millipore, Billerica, MA, USA), followed by sequential hybridization at 46 °C with bDNA amplifier and 5'-dT(biotin)-conjugated label probe. Unbound materials were washed from beads (complexed with probe set and mRNA) through alternating filtration and the addition of wash buffer (0.1xSSC, 0.03% lithium lauryl sulfate). Two washes were performed after each hybridization step. After a final wash, Streptavidin conjugated R-Phycoerythrin (SAPE) was added and was incubated at room temperature for 30 min. The beads were washed to remove unbound SAPE, followed by analysis with Luminex^{100}IS system (Luminex) or a Bio-Plex system (Bio-Rad). The level of SAPE fluorescence measured from each bead is proportional to the number of mRNA transcripts captured by the bead.

Cell lysates for evaluation of the cytokine panel

[0203] U937 cells (American Type Culture Collection) at a density around 1-2x10^5 cells/ml were allowed to differentiate in SFM media (GIBCO) with 100 nM Phorbol-12-myrstyl-13-acetate (PMA, Sigma) for 48 hrs. Differentiated cells, which become adherent, were stimulated with lipopolysaccharide (LPS, Sigma) at a concentration of 1μg/ml in RPMI growth media (GIBCO) with 10% FBS for 4 hours. After cell counting, the cells were directly lysed in the culture flask by the addition of Lysis Mixture to the culture media and incubated at 37°C for 15 minutes.

Cell lysates for evaluation of the apoptosis panel

[0204] HeLa cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) at an approximate density of 1-2x10^6 cells/ml and were treated with recombinant human TNFα (1 ng/ml, R&D Systems) or 1% BSA/PBS vehicle control. Both TNFα-treated and vehicle-treated cells were harvested at 1, 3 and 6 hour post-treatment by the addition of Lysis Mixture directly to the culture flasks in order to generate cell lysates suitable for both single-plex and multiplex assays.

Data analysis & statistics

[0205] Three replicate assays (n=3) were performed for all described experimental samples unless noted otherwise. For all samples, background signal levels in the absence of target mRNAs were determined and subtracted from signals obtained in the presence of target mRNAs. The correlation between the single-plex and multiplex bDNA assay was assessed through matched pair analysis using JMP (SAS Institute). Statistical significance of biological studies was tested using student's t-test or ANOVA where appropriate (P<0.01).

Claims

1. A method of detecting ten or more nucleic acids of interest, the method comprising:

a) providing a sample comprising or suspected of comprising the nucleic acids of interest;
b) providing i) a pooled population of particles, the population comprising ten or more subsets of particles, a plurality of the particles in each subset being distinguishable from a plurality of the particles in every other

subset, and the particles in each subset having associated therewith a different capture probe or ii) a solid support comprising ten or more different capture probes, wherein each different capture probe is provided at a different selected position on the solid support;
c) providing ten or more subsets of n capture extenders, wherein n is at least two, wherein each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and wherein the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected subset of the particles or with a selected position on the solid support;
d) contacting the sample, the pooled population of particles or the solid support, and the subsets of n capture extenders;
e) hybridizing any nucleic acid of interest present in the sample to its corresponding subset of n capture extenders and hybridizing the subset of n capture extenders to its corresponding capture probe, whereby the hybridizing the nucleic acid of interest to the n capture extenders and the n capture extenders to the corresponding capture probe captures the nucleic acid on the subset of particles with which the capture extenders are associated or on the solid support at the selected position with which the capture extenders are associated,
wherein the hybridizing the subset of n capture extenders to the corresponding capture probe is performed at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and its corresponding capture probe,
wherein the n capture extenders in a subset hybridize to nonoverlapping polynucleotide sequences in the corresponding nucleic acid of interest; and
f) determining which subsets of particles have a nucleic acid of interest captured on the particles or which positions on the solid support have a nucleic acid of interest captured at that position, thereby indicating which of the nucleic acids of interest were present in the sample, by i) hybridizing one or more label extenders and a label probe system comprising a label to any nucleic acid of interest captured on the particles and identifying at least a portion of the particles from each subset and detecting the presence or absence of the label on those particles or ii) hybridizing one or more label extenders and a label probe system comprising a label to any nucleic acid of interest captured on the solid support and detecting the presence or absence of the label at the selected positions on the solid support.

2. The method of claim **1,** wherein the ten or more nucleic acids of interest comprise 20 or more, 30 or more, 40 or more, 50 or more, 100 or more, 10^3 or more, or 10^4 or more nucleic acids of interest.

3. The method of claim **1,** wherein a pooled population of particles is provided, wherein the ten or more subsets of particles comprise 20 or more, 30 or more, 40 or more, or 50 or more subsets of particles, and wherein the ten or more subsets of n capture extenders comprise 20 or more, 30 or more, 40 or more, or 50 or more subsets of n capture extenders.

4. The method of claim **1,** wherein the particles are microspheres.

5. The method of claim **4,** wherein the microspheres of each subset are distinguishable from those of the other subsets on the basis of their fluorescent emission spectrum, their diameter, or a combination thereof.

6. The method of claim **1,** wherein n is at least three.

7. The method of claim **6,** wherein n is at least five.

8. The method of claim **1,** wherein n is at most ten.

9. The method of claim **1,** wherein each capture extender comprises a polynucleotide sequence C-1 that is complementary to a polynucleotide sequence C-2 in its corresponding capture probe, and wherein C-1 and C-2 are 20 nucleotides or less in length.

10. The method of claim **9,** wherein C-1 and C-2 are between 9 and 17 nucleotides in length.

11. The method of claim **10,** wherein C-1 and C-2 are between 12 and 15 nucleotides in length.

12. The method of claim **1,** wherein the hybridization temperature is about 5˚C or more greater than the T_m.

13. The method of claim **12,** wherein the hybridization temperature is about 7˚C or more, about 10˚C or more, about 12˚C or more, about 15˚C or more, about 17˚C or more, or about 20˚C or more greater than the T_m.

14. The method of claim **1,** wherein contacting the sample, the pooled population of particles or the solid support, and the subsets of n capture extenders comprises combining the sample with the subsets of n capture extenders to form a mixture, and then combining the mixture with the pooled population of particles or the solid support.

15. The method of claim **1,** wherein hybridizing one or more label extenders and a label probe system to any nucleic acid of interest captured on the particles or the solid support comprises providing ten or more subsets of one or more label extenders, wherein each subset of label extenders is capable of hybridizing to one of the nucleic acids of interest; hybridizing any nucleic acid of interest captured on the particles or the solid support to its corresponding subset of label extenders; and hybridizing the label probe system to the label extenders.

16. The method of claim **15,** wherein the label probe system comprises an amplification multimer and a plurality of label probes, wherein the amplification multimer is capable of hybridizing to a label extender and to a plurality of label probes.

17. The method of claim **16,** wherein the label probe comprises the label.

18. The method of claim **15,** wherein hybridizing any nucleic acid of interest captured on the particles or the solid support to its corresponding subset of label extenders comprises hybridizing any nucleic acid of interest present in the sample to its corresponding subset of label extenders and to its corresponding subset of n capture extenders, then hybridizing the subset of n capture extenders to its corresponding capture probe.

19. The method of claim **1,** wherein the label is a fluorescent label, and wherein detecting the presence of the label on the particles or the solid support comprises detecting a fluorescent signal from the label.

20. The method of claim **1,** wherein detecting the presence of the label on the particles or the solid support comprises measuring an intensity of a signal from the label, the method comprising correlating the intensity of the signal with a quantity of the corresponding nucleic acid of interest present.

21. The method of claim **1,** comprising separating materials not captured on the particles or the solid support from the particles or the solid support.

22. The method of claim **1,** wherein the sample was derived from one or more of: an animal, a human, a plant, a cultured cell, a virus, a bacterium, a pathogen, or a microorganism.

23. The method of claim **1,** wherein the sample comprises one or more of: a cell lysate, an intercellular fluid, a bodily fluid, or a conditioned culture medium.

24. The method of claim **1,** wherein the sample is derived from one or more of: a tissue, a biopsy, or a tumor.

25. The method of claim **1,** wherein the nucleic acids of interest are derived from one or more of: an animal, a human, a plant, a cultured cell, a microorganism, a virus, a bacterium, or a pathogen.

26. The method of claim **1,** wherein the ten or more nucleic acids of interest comprise ten or more mRNAs.

27. The method of claim **1,** wherein at least one of the nucleic acids of interest is present in the sample in a non-zero amount of 200 amol or less, 150 amol or less, 100 amol or less, 50 amol or less, 10 amol or less, 1 amol or less, or 0.1 amol or less.

28. The method of claim **1,** wherein the sample comprises a first nucleic acid of interest, and wherein at least 30%, at least 50%, at least 80%, at least 90%, or at least 95% of a total amount of the first nucleic acid present in the sample is captured on a first subset of particles or at a first selected position on the solid support.

29. The method of claim **1,** wherein the sample comprises or is suspected of comprising a first nucleic acid of interest and a second nucleic acid, the second nucleic acid having a polynucleotide sequence which is 95% or more identical to that of the first nucleic acid, wherein the first nucleic acid, if present in the sample, is captured on a first subset

of particles or at a first selected position on the solid support, and wherein the second nucleic acid comprises 1% or less of a total amount of nucleic acid captured on the first subset of particles or at the first selected position.

30. The method of claim **1,** wherein the sample comprises a first nucleic acid of interest and a second nucleic acid, the first nucleic acid being a first splice variant and the second nucleic acid being a second splice variant, wherein a first subset of n capture extenders is capable of hybridizing to the first splice variant, of which at most n-1 capture extenders are capable of hybridizing to the second splice variant.

31. The method of claim **30,** wherein hybridization of the n capture extenders to the first splice variant captures the first splice variant on a first subset of particles or at a first selected position on the solid support while hybridization of the at most n-1 capture extenders to the second splice variant does not capture the second splice variant on the first subset of particles or at the first selected position.

32. The method of claim **1,** wherein the label probe system comprises an amplification multimer and a plurality of label probes, wherein the amplification multimer is capable of hybridizing to the label extender and to the plurality of label probes, and wherein the amplification multimer and label probes comprise only naturally occurring bases A, C, G, T, and/or U; or wherein the label probe system comprises a preamplifier, a plurality of amplification multimers, and a plurality of label probes, wherein the preamplifier is capable of hybridizing to the label extender and the amplification multimers are capable of hybridizing to the preamplifier and to the plurality of label probes, and wherein the preamplifier, amplification multimer, and label probes comprise only naturally occurring bases A, C, G, T, and/or U.

33. A composition for detecting ten or more nucleic acids of interest, the composition comprising:

a) i) ten or more subsets of particles, a plurality of the particles in each subset being distinguishable from a plurality of the particles in every other subset, and the particles in each subset having associated therewith a different capture probe or ii) a solid support comprising ten or more different capture probes, wherein each different capture probe is provided at a different selected position on the solid support;
b) ten or more subsets of n capture extenders, wherein n is at least two, wherein each subset of n capture extenders is capable of hybridizing to one of the nucleic acids of interest, and wherein the capture extenders in each subset are capable of hybridizing to one of the capture probes and thereby associating each subset of n capture extenders with a selected subset of the particles or with a selected position on the solid support, wherein each subset of capture extenders is capable of hybridizing to the corresponding nucleic acid of interest and to the corresponding capture probe at a hybridization temperature which is greater than a melting temperature T_m of a complex between each individual capture extender and the capture probe;
c) ten or more subsets of one or more label extenders; and
d) a label probe system comprising a label.

34. The composition of claim **33,** packaged in one or more containers.

35. The composition of claim **33,** wherein the label probe system comprises an amplification multimer and a plurality of label probes, wherein the amplification multimer is capable of hybridizing to the label extender and to the plurality of label probes, and wherein the amplification multimer and label probes comprise only naturally occurring bases A, C, G, T, and/or U; or wherein the label probe system comprises a preamplifier, a plurality of amplification multimers, and a plurality of label probes, wherein the preamplifier is capable of hybridizing to the label extender and the amplification multimers are capable of hybridizing to the preamplifier and to the plurality of label probes, and wherein the preamplifier, amplification multimer, and label probes comprise only naturally occurring bases A, C, G, T, and/or U.

Patentansprüche

1. Verfahren zur Detektion von zehn oder mehr Nucleinsäuren von Interesse, wobei das Verfahren Folgendes umfasst:

a) das Bereitstellen einer Probe, die die Nucleinsäuren von Interesse umfasst oder vermutlich umfasst;
b) das Bereitstellen von i) einer vereinigten Population von Partikeln, wobei die Population zehn oder mehr Untergruppen von Partikeln umfasst, wobei eine Vielzahl von Partikeln in jeder Untergruppe von einer Vielzahl von Partikeln in jeder anderen Untergruppe unterscheidbar ist und mit den Partikeln in jeder Untergruppe eine jeweils andere Fangsonde verbunden ist, oder ii) einer Festphase, die zehn oder mehr verschiedene Fangsonden umfasst, worin jede der verschiedenen Fangsonden an einer anderen ausgewählten Position auf der

Festphase bereitgestellt wird;

c) das Bereitstellen von zehn oder mehr Untergruppen von n Fangerweiterungen, worin n zumindest 2 ist, worin jede Untergruppe von n Fangerweiterungen in der Lage ist, an eine der Nucleinsäuren von Interesse zu hybridisieren, und worin die Fangerweiterungen in jeder Untergruppe in der Lage sind, an eine der Fangsonden zu hybridisieren, wodurch jede Untergruppe von n Fangerweiterungen mit einer ausgewählten Untergruppe der Partikel oder mit einer ausgewählten Position auf der Festphase verbunden wird;

d) das Kontaktieren der Probe, der vereinigten Population von Partikeln oder der Festphase und der Untergruppe von n Fangerweiterungen;

e) das Hybridisieren einer Nucleinsäure von Interesse, die in der Probe vorhanden ist, an ihre entsprechende Untergruppe von n Fangerweiterungen und das Hybridisieren der Untergruppe von n Fangerweiterungen an die jeweilige Fangsonde, wodurch das Hybridisieren der Nucleinsäure von Interesse an die n Fangerweiterungen und jener der n Fangerweiterungen an die entsprechende Fangsonde die Nucleinsäure auf der Untergruppe von Partikeln, mit der die Fangerweiterungen verbunden sind, oder auf der Festphase an der ausgewählten Position, mit der die Fangerweiterungen verbunden sind, einfängt,

worin das Hybridisieren der Untergruppe von n Fangerweiterungen an die entsprechende Fangsonde bei einer Hybridisierungstemperatur durchgeführt wird, die höher ist als die Schmelztemperatur T_m eines Komplexes zwischen jeder einzelnen Fangerweiterung und ihrer dazugehörigen Fangsonde,

worin die n Fangerweiterungen in einer Untergruppe an nicht überlappende Polynucleotidsequenzen in einer entsprechenden Nucleinsäure von Interesse hybridisieren; und

f) das Bestimmen, auf den Partikeln welcher Untergruppen von Partikeln eine Nucleinsäure von Interesse eingefangen wurde oder an welchen Positionen auf der Festphase eine Nucleinsäure von Interesse eingefangen wurde, wodurch angezeigt wird, welche der Nucleinsäuren von Interesse in der Probe vorhanden waren, durch i) das Hybridisieren einer oder mehr Markierungserweiterungen und eines Markierungssondensystems, das eine Markierung umfasst, an eine auf den Partikeln gefangene Nucleinsäure von Interesse, das Identifizieren zumindest eines Teils der Partikel aus jeder Untergruppe und das Nachweisen der Gegenwart oder Abwesenheit der Markierung auf diesen Partikeln oder durch ii) das Hybridisieren einer oder mehr Markierungserweiterungen und eines Markierungssondensystems, das eine Markierung umfasst, an eine auf der Festphase gefangene Nucleinsäure von Interesse und das Nachweisen der Gegenwart oder Abwesenheit der Markierung an den ausgewählten Positionen auf der Festphase.

2. Verfahren nach Anspruch 1, worin die zehn oder mehr Nucleinsäuren von Interesse 20 oder mehr, 30 oder mehr, 40 oder mehr, 50 oder mehr, 100 oder mehr, 10^3 oder mehr oder 10^4 oder mehr Nucleinsäuren von Interesse umfasst.

3. Verfahren nach Anspruch 1, worin eine vereinigte Population von Partikeln bereitgestellt wird, worin die zehn oder mehr Untergruppen von Partikeln 20 oder mehr, 30 oder mehr, 40 oder mehr oder 50 oder mehr Untergruppen von Partikeln umfassen und worin die zehn oder mehr Untergruppen von n Fangerweiterungen 20 oder mehr, 30 oder mehr, 40 oder mehr oder 50 oder mehr Untergruppen von n Fangerweiterungen umfassen.

4. Verfahren nach Anspruch 1, worin die Partikel Mikrokugeln sind.

5. Verfahren nach Anspruch 4, worin die Mikrokugeln jeder Untergruppe von jenen der anderen Untergruppen anhand ihres Fluoreszenz-Emissionsspektrums, ihres Durchmessers oder einer Kombination davon unterscheidbar sind.

6. Verfahren nach Anspruch 1, worin n zumindest 3 ist.

7. Verfahren nach Anspruch 6, worin n zumindest 5 ist.

8. Verfahren nach Anspruch 1, worin n höchstens 10 ist.

9. Verfahren nach Anspruch 1, worin jede Fangerweiterung eine Polynucleotidsequenz C-1 umfasst, die zu einer Polynucleotidsequenz C-2 in ihrer entsprechenden Fangsonde komplementär ist, und worin C-1 und C-2 eine Länge von 20 Nucleotiden oder weniger aufweisen.

10. Verfahren nach Anspruch 9, worin C-1 und C-2 zwischen 9 und 17 Nucleotiden lang sind.

11. Verfahren nach Anspruch 10, worin C-1 und C-2 zwischen 12 und 15 Nucleotiden lang sind.

12. Verfahren nach Anspruch 1, worin die Hybridisierungstemperatur um etwa 5 °C oder mehr höher ist als die T_m.

13. Verfahren nach Anspruch 12, worin die Hybridisierungstemperatur um etwa 7 °C oder mehr, um etwa 10 °C oder mehr, um etwa 12 °C oder mehr, um etwa 15 °C oder mehr, um etwa 17 °C oder mehr oder um etwa 20 °C oder mehr höher ist als die T_m.

14. Verfahren nach Anspruch 1, worin das Kontaktieren der Probe, der vereinigten Population von Partikeln oder der Festphase und der Untergruppen von n Fangerweiterungen das Kombinieren der Probe mit den Untergruppen von n Fangerweiterungen, um ein Gemisch zu bilden, und das anschließende Kombinieren des Gemischs mit der vereinigten Population von Partikeln oder Festphase umfasst.

15. Verfahren nach Anspruch 1, worin das Hybridisieren von einer oder mehr Markierungserweiterungen und eines Markierungssondensystems an eine auf den Partikeln oder der Festphase eingefangene Nucleinsäure von Interesse das Bereitstellen von zehn oder mehr Untergruppen von einer oder mehr Markierungserweiterungen umfasst, worin jede Untergruppe von Markierungserweiterungen in der Lage ist, an eine der Nucleinsäuren von Interesse zu hybridisieren, das Hybridisieren einer auf den Partikeln oder der Festphase gefangenen Nucleinsäure von Interesse an die entsprechende Untergruppe von Markierungserweiterungen und das Hybridisieren des Markierungssondensystems an die Markierungserweiterungen umfasst.

16. Verfahren nach Anspruch 15, worin das Markierungssondensystem ein Amplifikationsmultimer und eine Vielzahl von Markierungssonden umfasst, worin das Amplifikationsmultimer in der Lage ist, an eine Markierungserweiterung und an eine Vielzahl von Markierungssonden zu hybridisieren.

17. Verfahren nach Anspruch 16, worin die Markierungssonde die Markierung umfasst.

18. Verfahren nach Anspruch 15, worin das Hybridisieren einer auf den Partikeln oder der Festphase gefangenen Nucleinsäure von Interesse an ihre entsprechende Untergruppe von Markierungserweiterungen das Hybridisieren einer in der Probe vorhandenen Nucleinsäure von Interesse an ihre entsprechende Untergruppe von Markierungserweiterungen und an ihre entsprechende Untergruppe von n Fangerweiterungen und das anschließende Hybridisieren der Untergruppe von n Fangerweiterungen an die entsprechende Fangsonde umfasst.

19. Verfahren nach Anspruch 1, worin die Markierung eine fluoreszierende Markierung ist und worin das Nachweisen der Gegenwart der Markierung auf den Partikeln oder der Festphase das Detektieren eines fluoreszierenden Signals von der Markierung umfasst.

20. Verfahren nach Anspruch 1, worin das Nachweisen der Gegenwart der Markierung auf den Partikeln oder der Festphase das Messen der Intensität eines Signals von der Markierung umfasst, wobei das Verfahren das Korrelieren der Intensität des Signals mit der vorhandenen Menge der entsprechenden Nucleinsäure von Interesse umfasst.

21. Verfahren nach Anspruch 1, umfassend das Trennen von Materialien, die nicht auf den Partikeln oder der Festphase gefangen sind, von den Partikeln oder der Festphase.

22. Verfahren nach Anspruch 1, worin die Probe von einem oder mehreren von einem Tier, einem Menschen, einer Pflanze, einer kultivierten Zelle, einem Virus, einem Bakterium, einem Pathogen oder einem Mikroorganismus stammt.

23. Verfahren nach Anspruch 1, worin die Probe eines oder mehrere von einem Zelllysat, einer interzellulären Flüssigkeit, einer Körperflüssigkeit oder einem konditionierten Kulturmedium umfasst.

24. Verfahren nach Anspruch 1, worin die Probe von einem oder mehreren von einem Gewebe, einer Biopsie und einem Tumor stammt.

25. Verfahren nach Anspruch 1, worin die Nucleinsäuren von Interesse von einem oder mehreren von einem Tier, einem Menschen, einer Pflanze, einer kultivierten Zelle, einem Mikroorganismus, einem Virus, einem Bakterium oder einem Pathogen stammt.

26. Verfahren nach Anspruch 1, worin die zehn oder mehr Nucleinsäuren von Interesse zehn oder mehr mRNAs umfassen.

27. Verfahren nach Anspruch 1, worin zumindest eine der Nucleinsäuren von Interesse in der Probe in einer Nicht-Null-

Menge von 200 amol oder weniger, 150 amol oder weniger, 100 amol oder weniger, 50 amol oder weniger, 10 amol oder weniger, 1 amol oder weniger oder 0,1 amol oder weniger vorhanden ist.

28. Verfahren nach Anspruch 1, worin die Probe eine erste Nucleinsäure von Interesse umfasst und worin zumindest 30 %, zumindest 50 %, zumindest 80 %, zumindest 90 % oder zumindest 95 % der in der Probe vorhandenen Gesamtmenge der ersten Nucleinsäure auf einer ersten Untergruppe von Partikeln oder an einer ersten ausgewählten Position der Festphase gefangen ist.

29. Verfahren nach Anspruch 1, worin die Probe eine erste Nucleinsäure von Interesse und eine zweite Nucleinsäure umfasst oder vermutlich umfasst, wobei die zweite Nucleinsäure eine Polynucleotidsequenz aufweist, die zu 95 % oder mehr mit jener der ersten Nucleinsäure identisch ist, worin die erste Nucleinsäure, wenn sie in der Probe vorhanden ist, auf einer ersten Untergruppe von Partikeln oder an einer ersten ausgewählten Position der Festphase gefangen ist und worin die zweite Nucleinsäure 1 % oder weniger der Gesamtmenge an auf der ersten Untergruppe von Partikeln oder der ersten ausgewählten Position gefangenen Nucleinsäuren umfasst.

30. Verfahren nach Anspruch 1, worin die Probe eine erste Nucleinsäure von Interesse und eine zweite Nucleinsäure umfasst, wobei die erste Nucleinsäure eine erste Spleißvariante ist und die zweite Nucleinsäure eine zweite Spleißvariante ist, worin eine erste Untergruppe von n Fangerweiterungen in der Lage ist, an die erste Spleißvariante zu hybridisieren, wovon höchstens n - 1 Fangerweiterungen in der Lage sind, an die zweite Spleißvariante zu hybridisieren.

31. Verfahren nach Anspruch 30, worin durch die Hybridisierung der n Fangerweiterungen an die erste Spleißvariante die erste Spleißvariante auf einer ersten Untergruppe von Partikeln oder an einer ersten ausgewählten Position auf der Festphase gefangen wird, während durch die Hybridisierung der höchstens n - 1 Fangerweiterungen an die zweite Spleißvariante die zweite Spleißvariante nicht auf der ersten Untergruppe von Partikeln oder an der ersten ausgewählten Position gefangen wird.

32. Verfahren nach Anspruch 1, worin das Markierungssondensystem ein Amplifikationsmultimer und eine Vielzahl von Markierungssonden umfasst, worin das Amplifikationsmultimer in der Lage ist, an die Markierungserweiterung und an die Vielzahl von Markierungssonden zu hybridisieren, und worin das Amplifikationsmultimer und die Markierungssonden nur die natürlich vorkommenden Basen A, C, G, T und/oder U umfassen; oder worin das Markierungssondensystem einen Voramplifikator, eine Vielzahl von Amplifikationsmultimeren und eine Vielzahl von Markierungssonden umfasst, worin der Voramplifikator in der Lage ist, an die Markierungserweiterung zu hybridisieren, und die Amplifikationsmultimere in der Lage sind, an den Voramplifikator und an die Vielzahl von Markierungssonden zu hybridisieren, und worin der Voramplifikator, das Amplifikationsmultimer und die Markierungssonden nur die natürlich vorkommenden Basen A, C, G, T und/oder U umfassen.

33. Zusammensetzung zur Detektion von zehn oder mehr Nucleinsäuren von Interesse, wobei die Zusammensetzung Folgendes umfasst:

a) i) zehn oder mehr Untergruppen von Partikeln, wobei eine Vielzahl von Partikeln in jeder Untergruppe von einer Vielzahl von Partikeln in jeder anderen Untergruppe unterscheidbar ist und mit den Partikel in jeder Untergruppe eine jeweils andere Fangsonde verbunden ist, oder ii) eine Festphase, die zehn oder mehr verschiedene Fangsonden umfasst, worin jede der verschiedenen Fangsonden an einer anderen ausgewählten Position auf der Festphase bereitgestellt wird;
b) zehn oder mehr Untergruppen von n Fangerweiterungen, worin n zumindest 2 ist, worin jede Untergruppe von n Fangerweiterungen in der Lage ist, an eine der Nucleinsäuren von Interesse zu hybridisieren, und worin die Fangerweiterungen in jeder Untergruppe in der Lage sind, an eine der Fangsonden zu hybridisieren, wodurch jede Untergruppe von n Fangerweiterungen mit einer ausgewählten Untergruppe der Partikel oder mit einer ausgewählten Position auf der Festphase verbunden wird,
worin jede Untergruppe von Fangerweiterungen in der Lage ist, bei einer Hybridisierungstemperatur, die höher ist als die Schmelztemperatur T_m eines Komplexes zwischen jeder einzelnen Fangerweiterung und der Fangsonde, an die entsprechende Nucleinsäure von Interesse und an die entsprechende Fangsonde zu hybridisieren;
c) zehn oder mehr Untergruppen von einer oder mehr Fangerweiterungen; und
d) ein Markierungssondensystem, das eine Markierung umfasst.

34. Zusammensetzung nach Anspruch 33, verpackt in einem oder mehr Behältern.

35. Zusammensetzung nach Anspruch 33, worin das Markierungssondensystem ein Amplifikationsmultimer und eine Vielzahl von Markierungssonden umfasst, worin das Amplifikationsmultimer in der Lage ist, an die Markierungserweiterung und an die Vielzahl von Markierungssonden zu hybridisieren, und worin das Amplifikationsmultimer und die Markierungssonden nur die natürlich vorkommenden Basen A, C, G, T und/oder U umfassen; oder worin das Markierungssondensystem einen Voramplifikator, eine Vielzahl von Amplifikationsmultimeren und eine Vielzahl von Markierungssonden umfasst, worin der Voramplifikator in der Lage ist, an die Markierungserweiterung zu hybridisieren, und die Amplifikationsmultimere in der Lage sind, an den Voramplifikator und an die Vielzahl von Markierungssonden zu hybridisieren, und worin der Voramplifikator, das Amplifikationsmultimer und die Markierungssonden nur die natürlich vorkommenden Basen A, C, G, T und/oder U umfassen.

Revendications

1. Procédé pour détecter au moins dix acides nucléiques d'intérêt, le procédé consistant à:

a) mettre à disposition un échantillon comprenant ou suspecté de comprendre les acides nucléiques d'intérêt;
b) mettre à disposition i) une population de particules en pool, la population comprenant au moins dix sous-ensembles de particules, une pluralité des particules dans chaque sous-ensemble pouvant être différenciée d'une pluralité des particules dans chaque autre sous-ensemble, et les particules dans chaque sous-ensemble étant associées à une sonde de capture différente ou ii) un support solide comprenant au moins dix sondes de capture différentes, où chaque sonde de capture différente est fournie au niveau d'une position différente sélectionnée sur le support solide;
c) mettre à disposition au moins dix sous-ensembles de n sondes d'extension de capture, où n est au moins deux, où chaque sous-ensemble de n sondes d'extension de capture est capable de s'hybrider à un des acides nucléiques d'intérêt, et où les sondes d'extension de capture dans chaque sous-ensemble sont capables de s'hybrider à une des sondes de capture et d'ainsi d'associer chaque sous-ensemble de n sondes d'extension de capture avec un sous-ensemble des particules sélectionné ou avec une position sélectionnée sur le support solide;
d) mettre en contact l'échantillon, la population de particules en pool ou le support solide, et les sous-ensembles de n sondes d'extension de capture;
e) hybrider un quelconque acide nucléique d'intérêt présent dans l'échantillon à son sous-ensemble de n sondes d'extension de capture correspondant et hybrider le sous-ensemble de n sondes d'extension de capture à sa sonde de capture correspondante, moyennant quoi l'hybridation de l'acide nucléique d'intérêt aux n sondes d'extension de capture et des n sondes d'extension de capture à la sonde de capture correspondante permet de capturer l'acide nucléique sur le sous-ensemble de particules auquel sont associées les sondes d'extension de capture, ou sur le support solide au niveau de la position sélectionnée à laquelle sont associées les sondes d'extension de capture, où l'hybridation du sous-ensemble de n sondes d'extension de capture à la sonde de capture correspondante est réalisée à une température d'hybridation qui est supérieure à la température de fusion T_m d'un complexe entre chaque sonde d'extension de capture individuelle et sa sonde de capture correspondante,
où les n sondes d'extension de capture dans un sous-ensemble s'hybrident à des séquences polynucléotidiques non chevauchantes dans l'acide nucléique d'intérêt correspondant; et
f) déterminer quels sont les sous-ensembles de particules où un acide nucléique d'intérêt est capturé sur les particules, ou quelles sont les positions sur le support solide où un acide nucléique est capturé au niveau de cette position, ce qui indique ainsi quels sont les acides nucléiques d'intérêt qui étaient présents dans l'échantillon, en i) hybridant une ou plusieurs sondes d'extension de marquage, et un système de sonde de marquage comprenant un marqueur, à un quelconque acide nucléique d'intérêt capturé sur les particules, et en identifiant au moins une partie des particules de chaque sous-ensemble et en détectant la présence ou l'absence du marqueur sur ces particules, ou ii) en hybridant une ou plusieurs sondes d'extension de marquage, et un système de sonde de marquage comprenant un marqueur, à un quelconque acide nucléique d'intérêt capturé sur le support solide, et en détectant la présence ou l'absence du marqueur au niveau des positions sélectionnées sur le support solide.

2. Procédé selon la revendication 1, dans lequel les au moins dix acides nucléiques d'intérêt comprennent au moins 20, au moins 30, au moins 40, au moins 50, au moins 100, au moins 10^3, ou au moins 10^4 acides nucléiques d'intérêt.

3. Procédé selon la revendication 1, dans lequel une population de particules en pool est mise à disposition, où les au moins dix sous-ensembles de particules comprennent au moins 20, au moins 30, au moins 40, au moins 50 sous-ensembles de particules, et où les au moins dix sous-ensembles de n sondes d'extension de capture comprennent au moins 20, au moins 30, au moins 40, ou au moins 50 sous-ensembles de n sondes d'extension de capture.

4. Procédé selon la revendication 1, dans lequel les particules sont des microsphères.

5. Procédé selon la revendication 4, dans lequel les microsphères de chaque sous-ensemble peuvent être différenciées de celles des autres sous-ensembles en se basant sur leur spectre d'émission de fluorescence, leur diamètre, ou une combinaison de ceux-ci.

6. Procédé selon la revendication 1, dans lequel n est au moins trois.

7. Procédé selon la revendication 6, dans lequel n est au moins cinq.

8. Procédé selon la revendication 1, dans lequel n est au maximum dix.

9. Procédé selon la revendication 1, dans lequel chaque sonde d'extension de capture comprend une séquence polynucléotidique C-1 qui est complémentaire à une séquence polynucléotidique C-2 dans sa sonde de capture correspondante, et où C-1 et C-2 ont une longueur de 20 nucléotides ou moins.

10. Procédé selon la revendication 9, dans lequel C-1 et C-2 ont une longueur comprise entre 9 et 17 nucléotides.

11. Procédé selon la revendication 10, dans lequel C-1 et C-2 ont une longueur comprise entre 12 et 15 nucléotides.

12. Procédé selon la revendication 1, dans lequel la température d'hybridation est au moins environ 5 °C supérieure à la T_m.

13. Procédé selon la revendication 12, dans lequel la température d'hybridation est au moins environ 7 °C, au moins environ 10°C, au moins environ 12 °C, au moins environ 15°C, au moins environ 17 °C, ou au moins environ 20 °C supérieure à la T_m.

14. Procédé selon la revendication 1, dans lequel la mise en contact de l'échantillon, de la population de particules en pool, ou du support solide, et des sous-ensembles de n sondes d'extension de capture consiste à combiner l'échantillon avec les sous-ensembles de n sondes d'extension de capture afin de former un mélange, et ensuite à combiner le mélange avec la population de particules en pool ou le support solide.

15. Procédé selon la revendication 1, dans lequel l'hybridation d'une ou de plusieurs sondes d'extension de marquage et d'un système de sonde de marquage à un quelconque acide nucléique d'intérêt capturé sur les particules ou le support solide consiste à fournir au moins dix sous-ensembles d'une ou de plusieurs sondes d'extension de marquage, où chaque sous-ensemble de sondes d'extension de marquage est capable de s'hybrider à un des acides nucléiques d'intérêt; hybrider un quelconque acide nucléique d'intérêt capturé sur les particules ou le support solide à son sous-ensemble de sondes d'extension de marquage correspondant; et hybrider le système de sonde de marquage aux sondes d'extension de marquage.

16. Procédé selon la revendication 15, dans lequel le système de sonde de marquage comprend un multimère d'amplification et une pluralité de sondes de marquage, où le multimère d'amplification est capable de s'hybrider à une sonde d'extension de marquage et à une pluralité de sondes de marquage.

17. Procédé selon la revendication 16, dans lequel la sonde de marquage comprend le marqueur.

18. Procédé selon la revendication 15, dans lequel l'hybridation d'un quelconque acide nucléique d'intérêt capturé sur les particules ou le support solide à son sous-ensemble de sondes d'extension de marquage correspondant consiste à hybrider un quelconque acide nucléique d'intérêt présent dans l'échantillon à son sous-ensemble de sondes d'extension de marquage correspondant et à son sous-ensemble de n sondes d'extension de capture correspondant, puis à hybrider le sous-ensemble de n sondes d'extension de capture à sa sonde de capture correspondante.

19. Procédé selon la revendication 1, dans lequel le marqueur est un marqueur fluorescent, et dans lequel la détection de la présence du marqueur sur les particules ou le support solide consiste à détecter un signal fluorescent émis par le marqueur.

20. Procédé selon la revendication 1, dans lequel la détection de la présence du marqueur sur les particules ou le support solide consiste à mesurer l'intensité d'un signal émis par le marqueur, le procédé consistant à corréler l'intensité du signal avec une quantité de l'acide nucléique d'intérêt présent correspondant.

21. Procédé selon la revendication 1, consistant à séparer les matériaux qui ne sont pas capturés sur les particules ou le support solide des particules ou du support solide.

22. Procédé selon la revendication 1, dans lequel l'échantillon a été dérivé d'un ou de plusieurs parmi: un animal, un humain, une plante, une cellule en culture, un virus, une bactérie, un agent pathogène, ou un micro-organisme.

23. Procédé selon la revendication 1, dans lequel l'échantillon comprend un ou plusieurs parmi: un lysat cellulaire, un liquide intercellulaire, un liquide corporel, ou un milieu de culture conditionné.

24. Procédé selon la revendication 1, dans lequel l'échantillon est dérivé d'un ou plusieurs parmi: un tissu, une biopsie, ou une tumeur.

25. Procédé selon la revendication 1, dans lequel les acides nucléiques d'intérêt sont dérivés d'un ou de plusieurs parmi: un animal, un humain, une plante, une cellule en culture, un micro-organisme, un virus, une bactérie, ou un agent pathogène.

26. Procédé selon la revendication 1, dans lequel les au moins dix acides nucléiques d'intérêt comprennent au moins dix ARNm.

27. Procédé selon la revendication 1, dans lequel au moins un des acides nucléiques d'intérêt est présent dans l'échantillon en une quantité autre que zéro d'au maximum 200 amol, d'au maximum 150 amol, d'au maximum 100 amol, d'au maximum 50 amol, d'au maximum 10 amol, d'au maximum 1 amol, ou d'au maximum 0,1 amol.

28. Procédé selon la revendication 1, dans lequel l'échantillon comprend un premier acide nucléique d'intérêt, et dans lequel au moins 30 %, au moins 50 %, au moins 80 %, au moins 90 %, ou au moins 95 % d'une quantité totale du premier acide nucléique présent dans l'échantillon est capturée sur un premier sous-ensemble de particules ou au niveau d'une première position sélectionnée sur le support solide.

29. Procédé selon la revendication 1, dans lequel l'échantillon comprend ou est suspecté de comprendre un premier acide nucléique d'intérêt et un deuxième acide nucléique, le deuxième acide nucléique ayant une séquence polynucléotidique qui est au moins 95 % identique à celle du premier acide nucléique, où le premier acide nucléique, s'il est présent dans l'échantillon, est capturé sur un premier sous-ensemble de particules ou au niveau d'une première position sélectionnée sur le support solide, et où le deuxième acide nucléique comprend au maximum 1 % d'une quantité totale d'acide nucléique capturée sur le premier sous-ensemble de particules ou au niveau de la première position sélectionnée.

30. Procédé selon la revendication 1, dans lequel l'échantillon comprend un premier acide nucléique d'intérêt et un deuxième acide nucléique, le premier acide nucléique étant un premier variant d'épissage et le deuxième acide nucléique étant un deuxième variant d'épissage, où un premier sous-ensemble de n sondes d'extension de capture est capable de s'hybrider au premier variant d'épissage, parmi lequel au maximum n-1 sondes d'extension de capture sont capables de s'hybrider au deuxième variant d'épissage.

31. Procédé selon la revendication 30, dans lequel l'hybridation des n sondes d'extension de capture au premier variant d'épissage permet de capturer le premier variant d'épissage sur un premier sous-ensemble de particules ou au niveau d'une première position sélectionnée sur le support solide, alors que l'hybridation des au maximum n-1 sondes d'extension de capture au deuxième variant d'épissage ne permet pas de capturer le deuxième variant d'épissage sur le premier sous-ensemble de particules ou au niveau de la première position sélectionnée.

32. Procédé selon la revendication 1, dans lequel le système de sonde de marquage comprend un multimère d'amplification et une pluralité de sondes de marquage, où le multimère d'amplification est capable de s'hybrider à la sonde d'extension de marquage et à la pluralité de sondes de marquage, et dans lequel le multimère d'amplification et les sondes de marquage comprennent uniquement les bases survenant à l'état naturel A, C, G, T, et/ou U; ou dans lequel le système de sonde de marquage comprend un pré-amplificateur, une pluralité de multimères d'amplification, et une pluralité de sondes de marquage, où le pré-amplificateur est capable de s'hybrider à la sonde d'extension de marquage et les multimères d'amplification sont capables de s'hybrider au pré-amplificateur et à la pluralité de sondes de marquage, et où le pré-amplificateur, le multimère d'amplification, et les sondes de marquage comprennent uniquement les bases survenant à l'état naturel A, C, G, T, et/ou U.

33. Composition pour détecter au moins dix acides nucléiques d'intérêt, la composition comprenant:

a) au moins dix sous-ensembles de particules, une pluralité des particules dans chaque sous-ensemble pouvant être différenciée d'une pluralité des particules dans chaque autre sous-ensemble, et les particules dans chaque sous-ensemble étant associées à une sonde de capture différente, ou ii) un support solide comprenant au moins dix sondes de capture différentes, où chaque sonde de capture différente est fournie au niveau d'une position différente sélectionnée sur le support solide;
b) au moins dix sous-ensembles de n sondes d'extension de capture, où n est au moins deux, où chaque sous-ensemble de n sondes d'extension de capture est capable de s'hybrider à un des acides nucléiques d'intérêt, et où les sondes d'extension de capture dans chaque sous-ensemble sont capables de s'hybrider à une des sondes de capture et d'ainsi associer chaque sous-ensemble de n sondes d'extension de capture à un sous-ensemble des particules sélectionné ou à une position sélectionnée sur le support solide,
où chaque sous-ensemble de sondes d'extension de capture est capable de s'hybrider à l'acide nucléique d'intérêt- correspondant et à la sonde de capture correspondante, à une température d'hybridation qui est supérieure à une température de fusion T_m d'un complexe entre chaque sonde d'extension de capture individuelle et la sonde de capture;
c) au moins dix sous-ensembles d'une ou de plusieurs sondes d'extension de marquage; et
d) un système de sonde de marquage comprenant un marqueur.

34. Composition selon la revendication 33, qui est conditionnée dans un ou plusieurs récipients.

35. Composition selon la revendication 33, dans laquelle le système de sonde de marquage comprend un multimère d'amplification et une pluralité de sondes de marquage, où le multimère d'amplification est capable de s'hybrider à la sonde d'extension de marquage et à la pluralité de sondes de marquage, et où le multimère d'amplification et les sondes de marquage comprennent uniquement les bases survenant à l'état naturel A, C, G, T, et/ou U; où dans laquelle le système de sonde de marquage comprend un pré-amplificateur, une pluralité de multimères d'amplification, et une pluralité de sondes de marquage, où le pré-amplificateur est capable de s'hybrider à la sonde d'extension de marquage et les multimères d'amplification sont capables de s'hybrider au pré-amplificateur et à la pluralité de sondes de marquage, et où le pré-amplificateur, le multimère d'amplification, et les sondes de marquage comprennent uniquement les bases survenant à l'état naturel A, C, G, T, et/ou U.

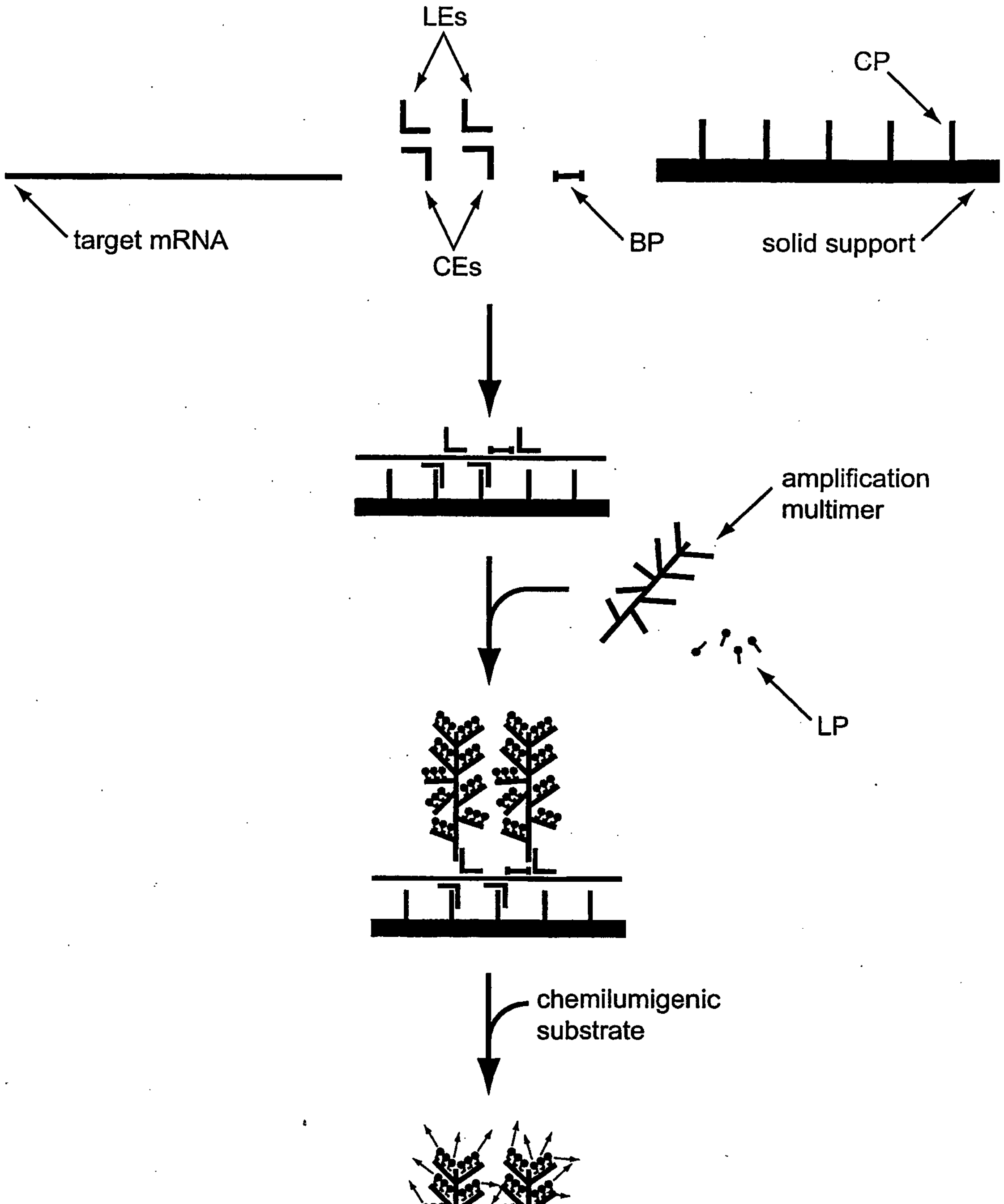

LEs
CP
target mRNA
CEs
BP
solid support
amplification multimer
LP
chemilumigenic substrate

Fig. 1

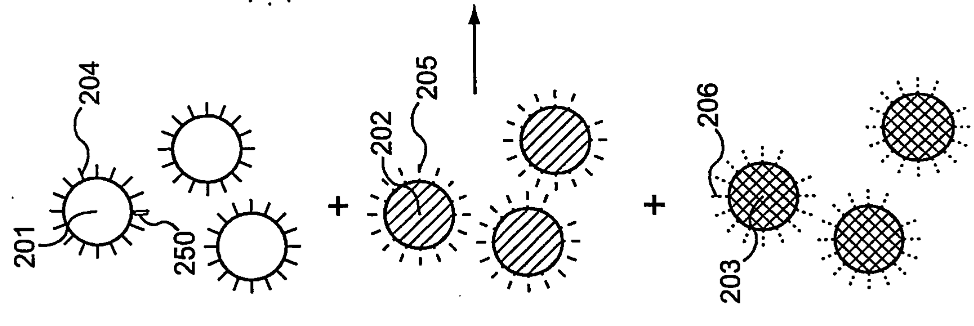


Fig. 2A

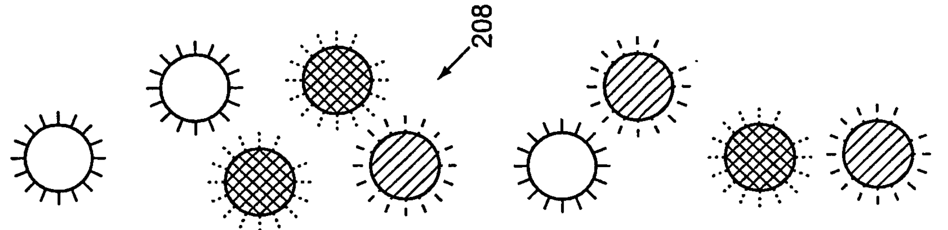


Fig. 2B

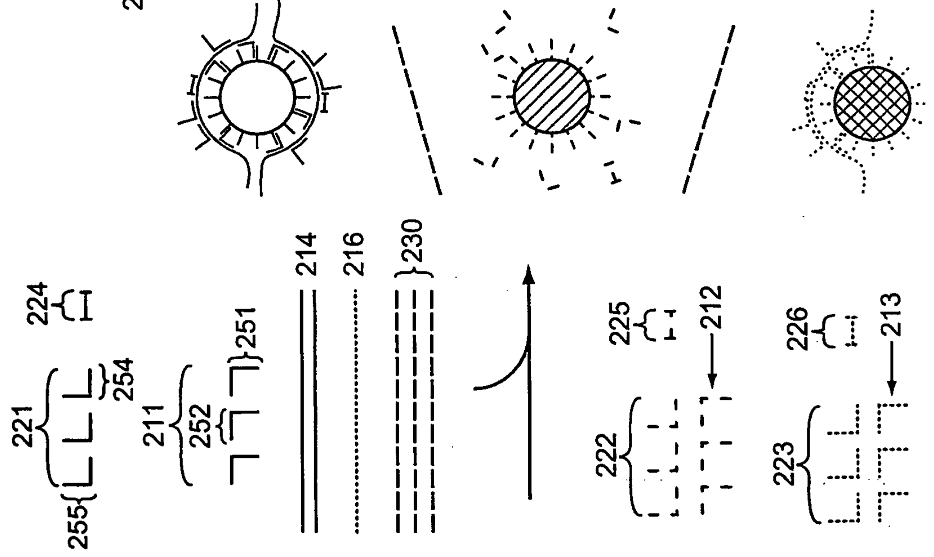


Fig. 2C

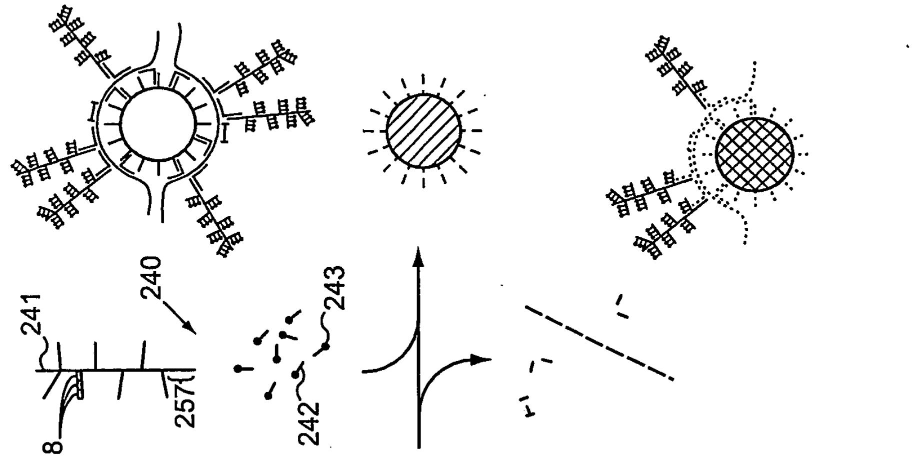


Fig. 2D

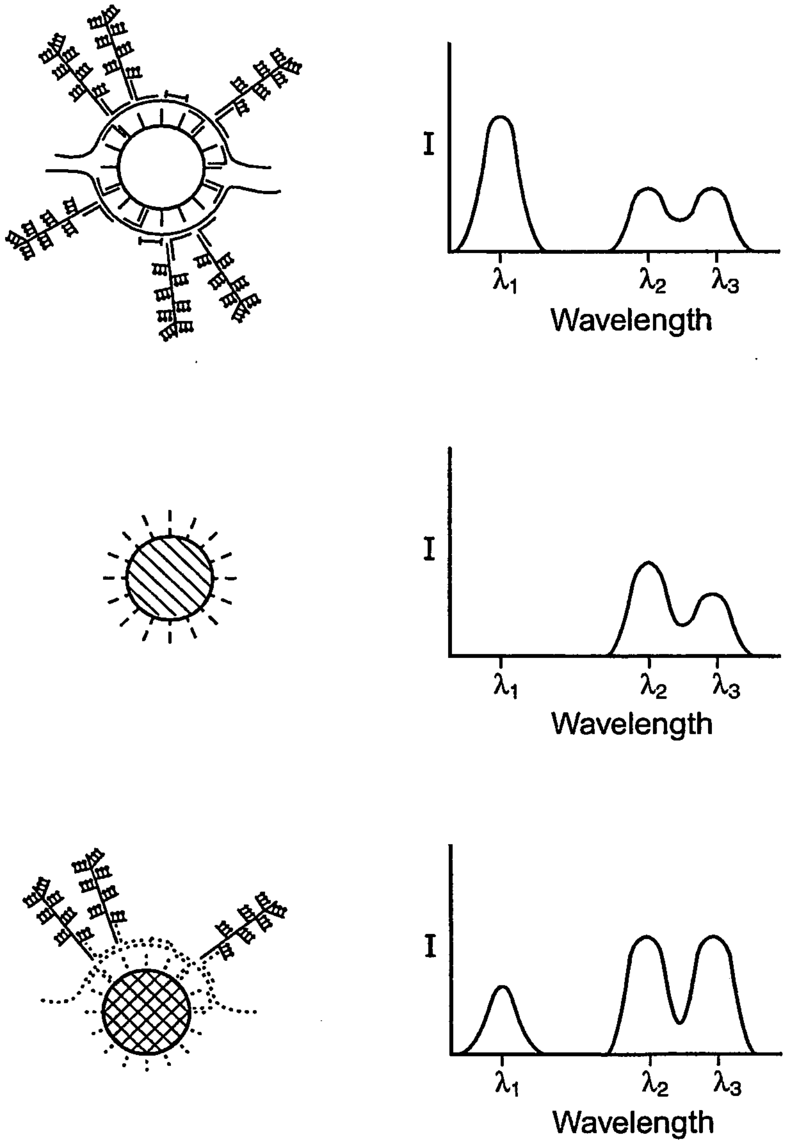
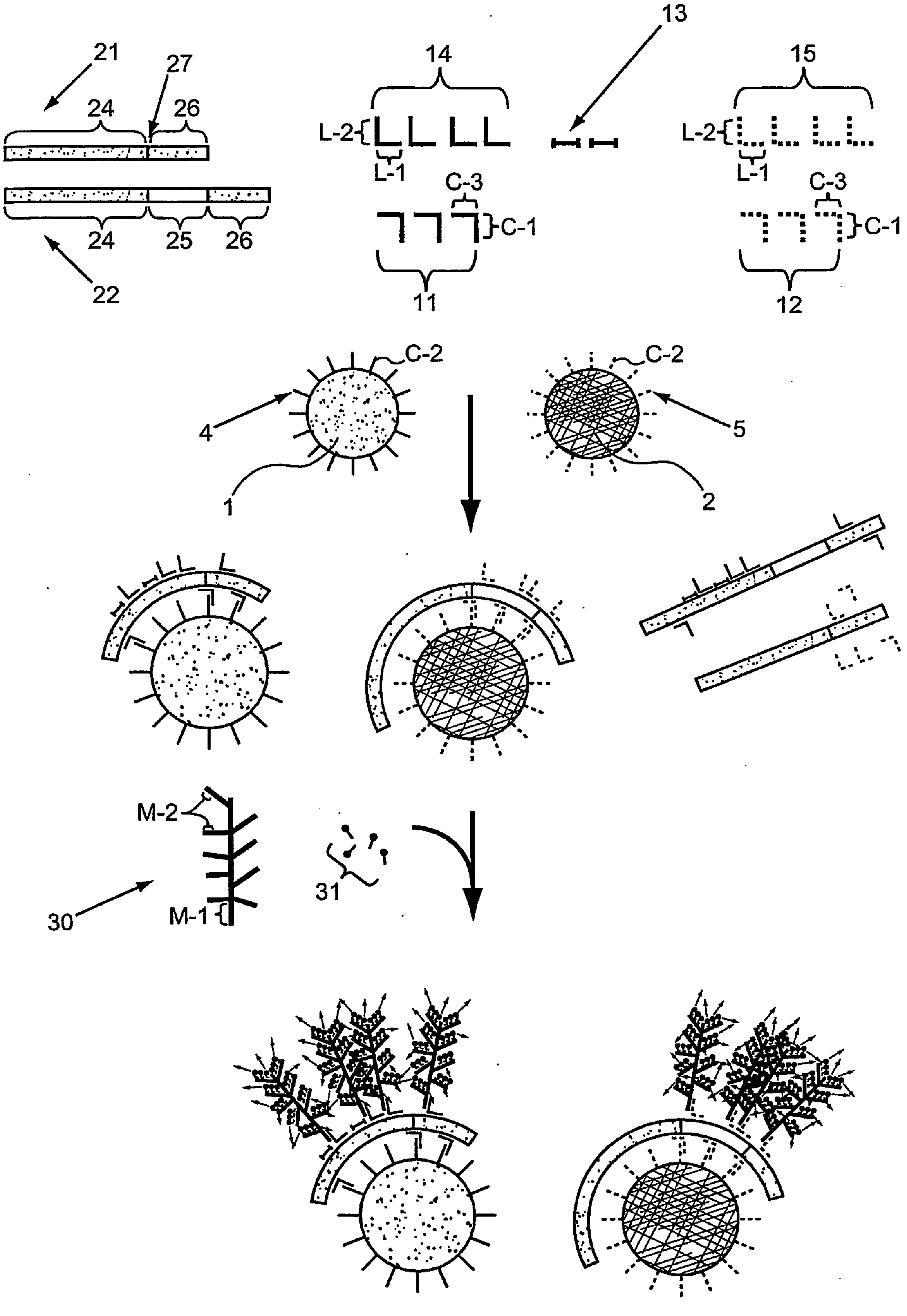

I
λ1
λ2
λ3
Wavelength
I
λ1
λ2
λ3
Wavelength
I
λ1
λ2
λ3
Wavelength

Fig. 2E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L-2
L-1
C-3
C-1
11
L-2
L-1
C-3
C-1
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C-2
4
1
C-2
5
2
M-2
30
M-1
31

Fig. 3

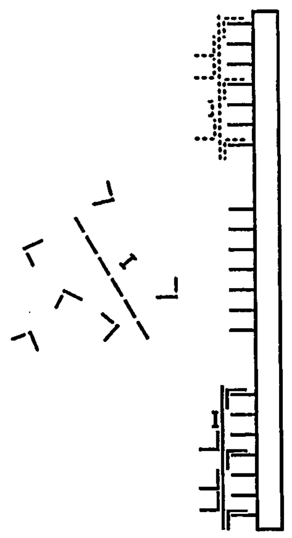


Fig. 4C

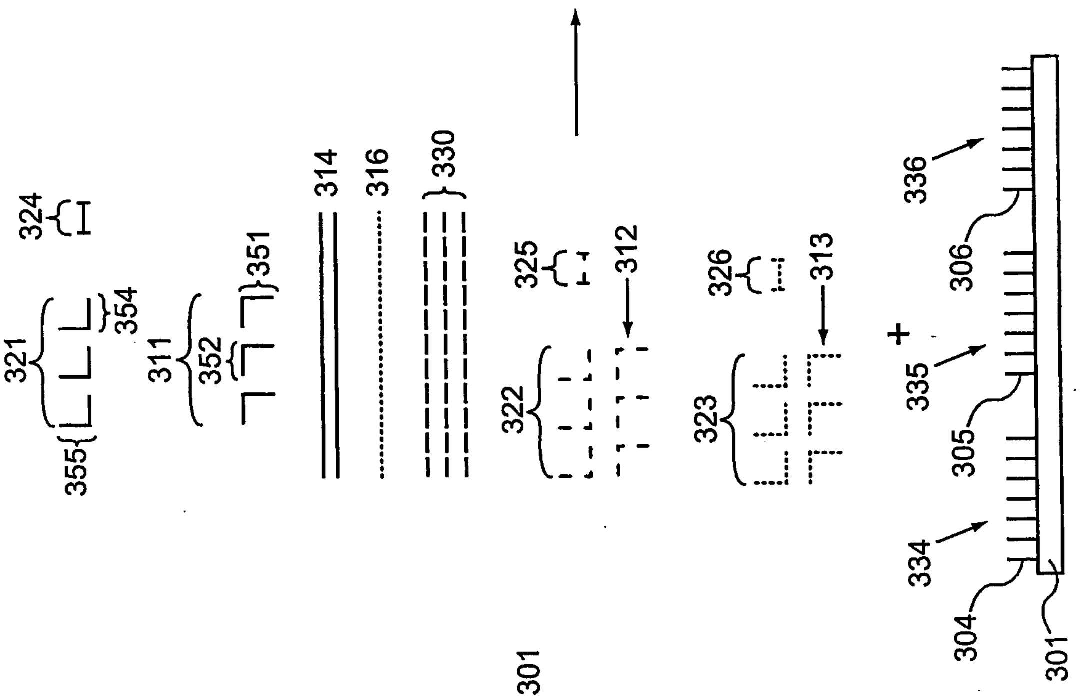


Fig. 4B

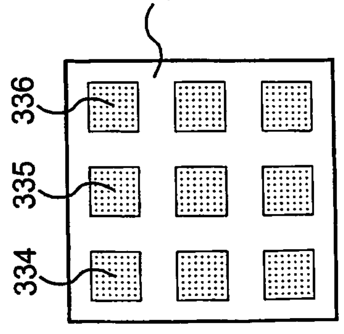


Fig. 4A

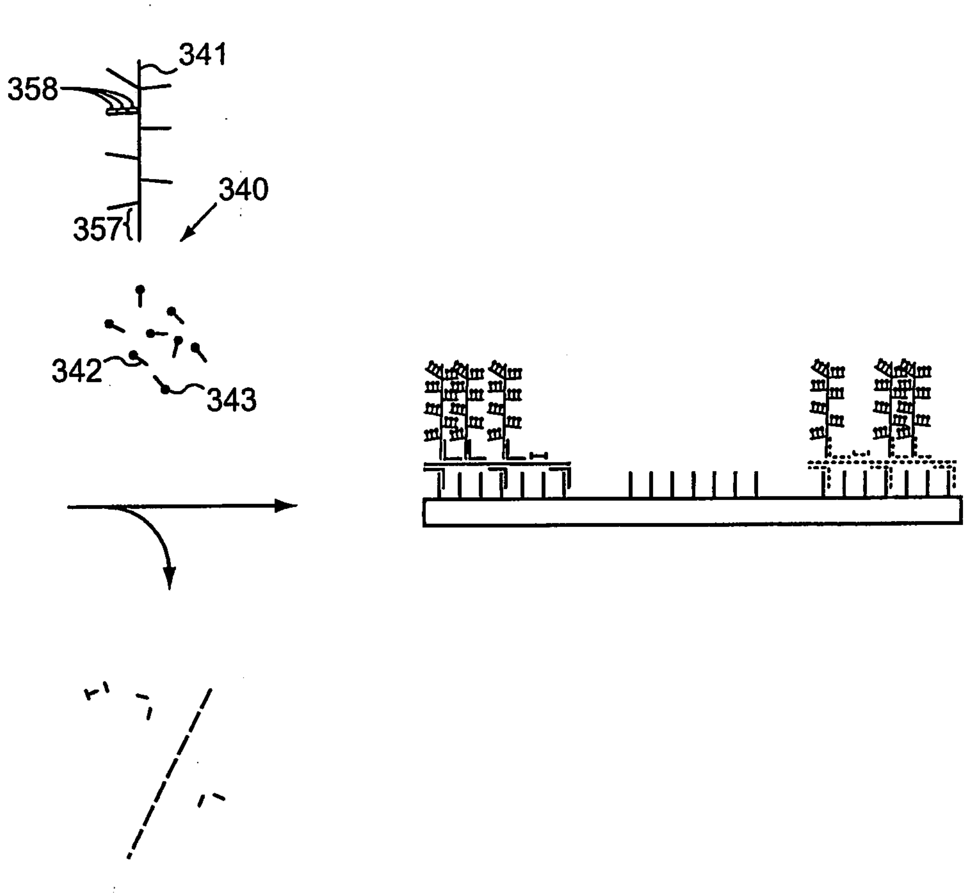


Fig. 4D

Fig. 5A

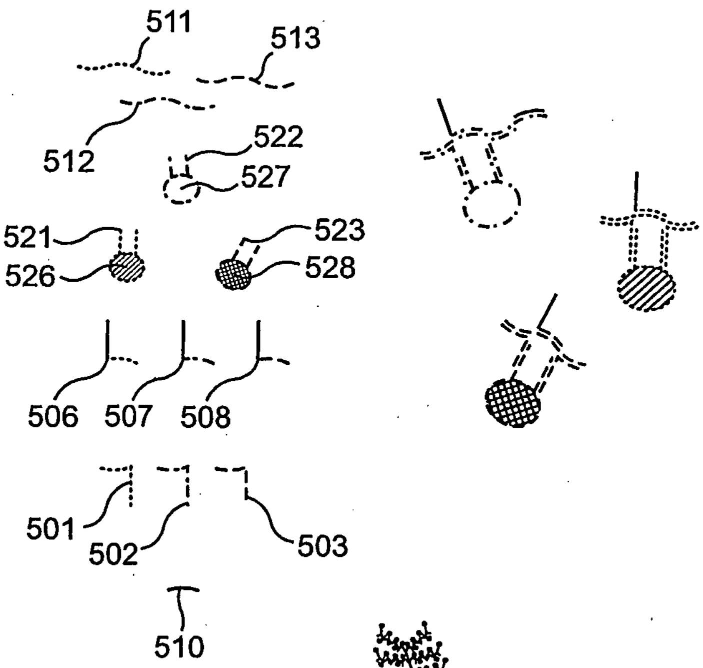


Fig. 5B

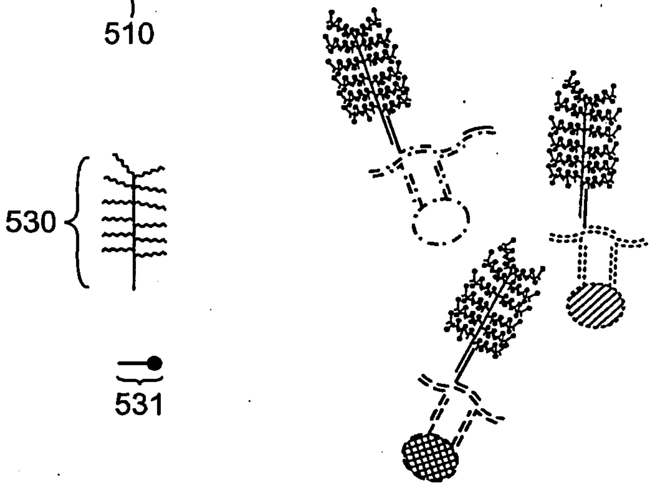
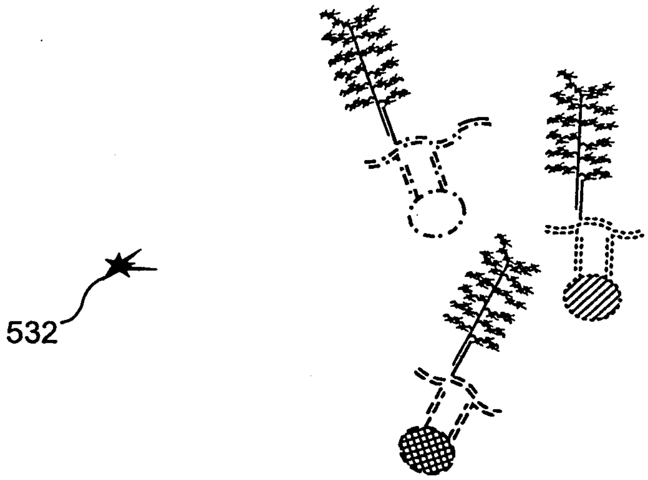


Fig. 5C



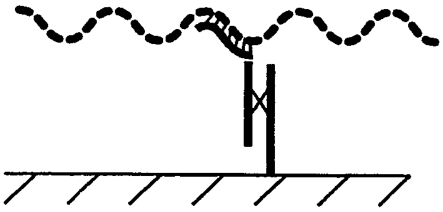


Fig. 6A

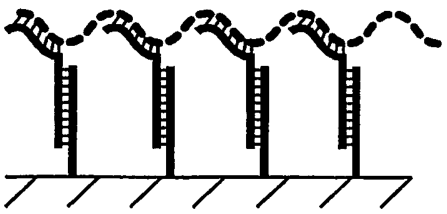


Fig. 6B

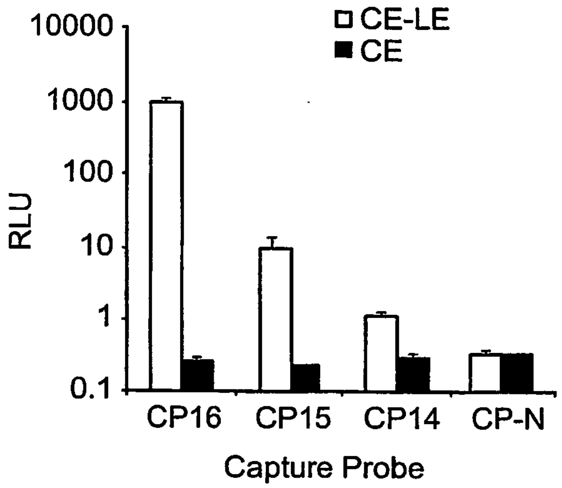


Fig. 6C

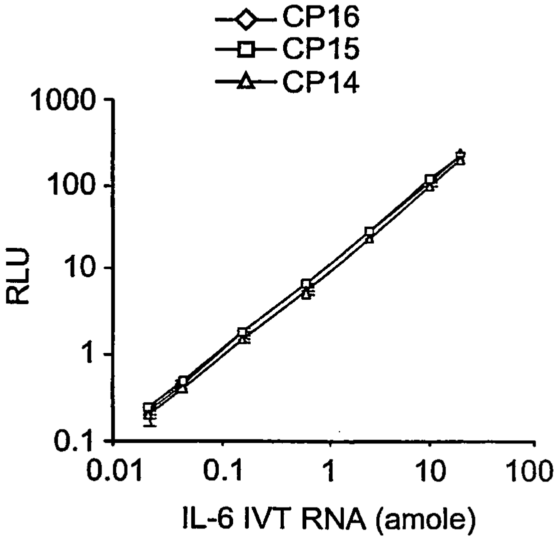


Fig. 6D

Fig. 7A

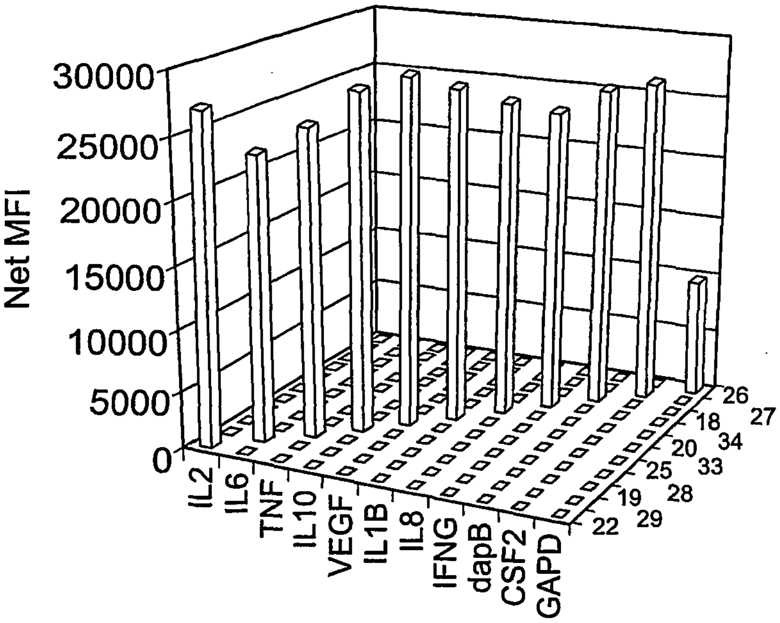


Fig. 7B

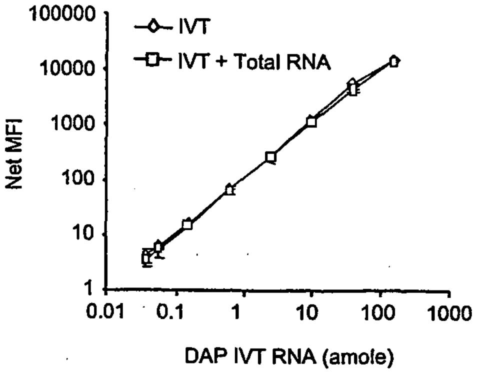
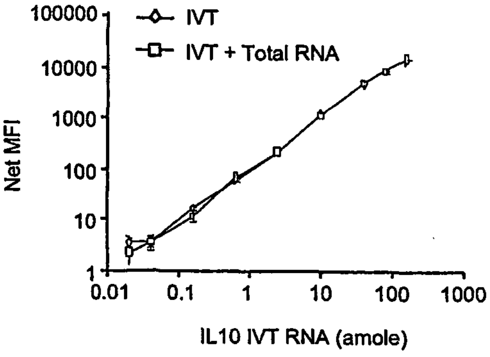
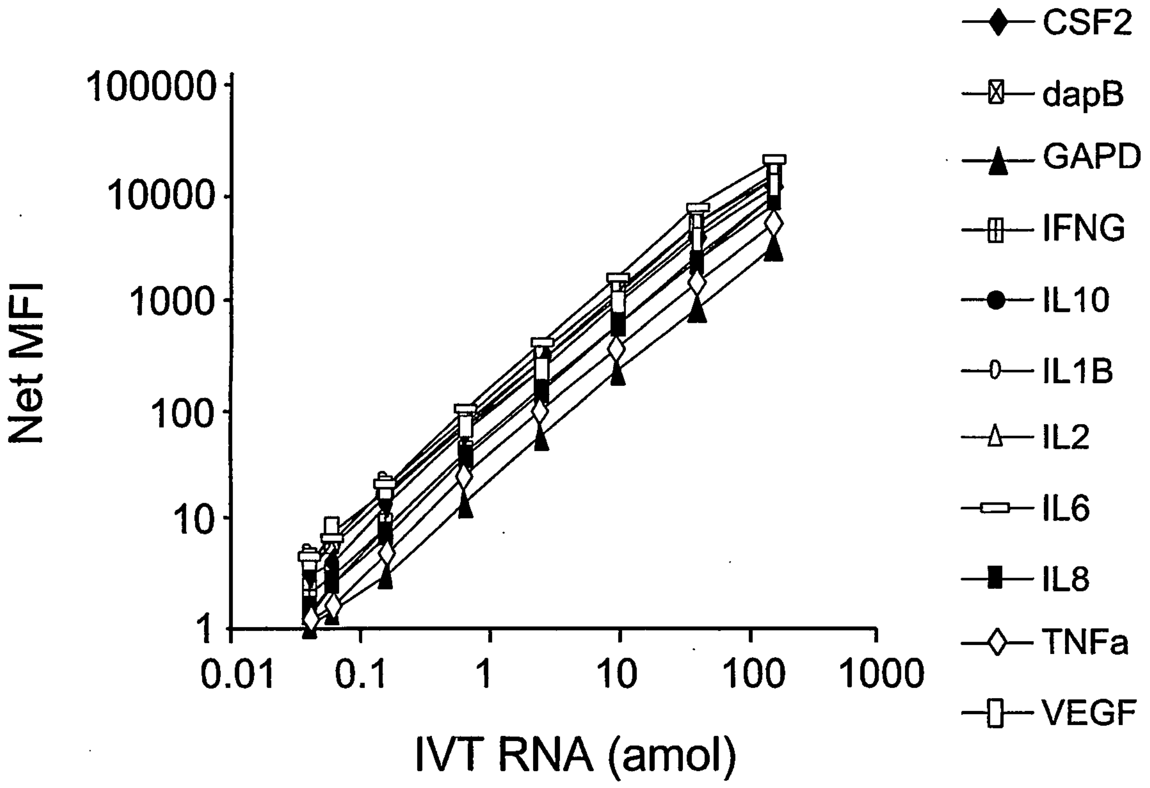


Fig. 7C

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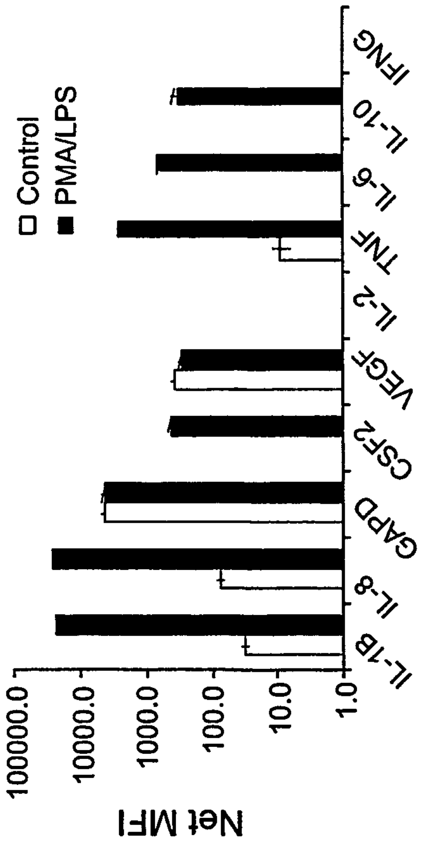
100000
10000
1000
100
10
1
0.01
0.1
1
10
100
1000
IVT RNA (amol)
CSF2
dapB
GAPD
IFNG
IL10
IL1B
IL2
IL6
IL8
TNFa
VEGF

Fig. 7D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10.0
1.0
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VEGF
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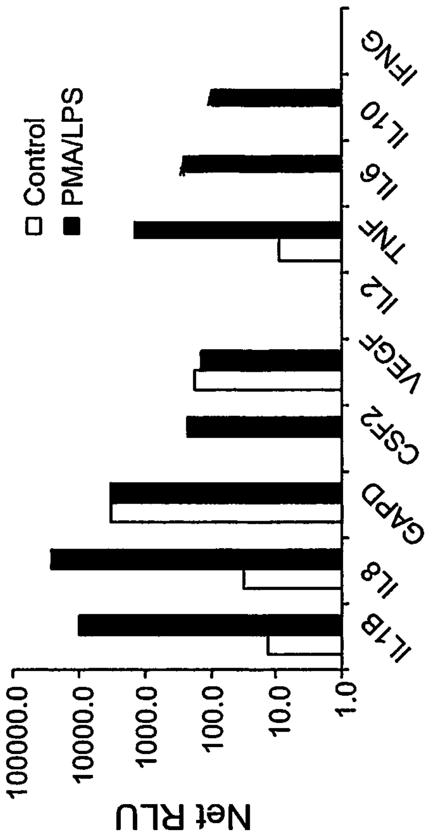
TNF
IL-6
IL-10
IFNG
☐ Control
■ PMA/LPS

Fig. 8A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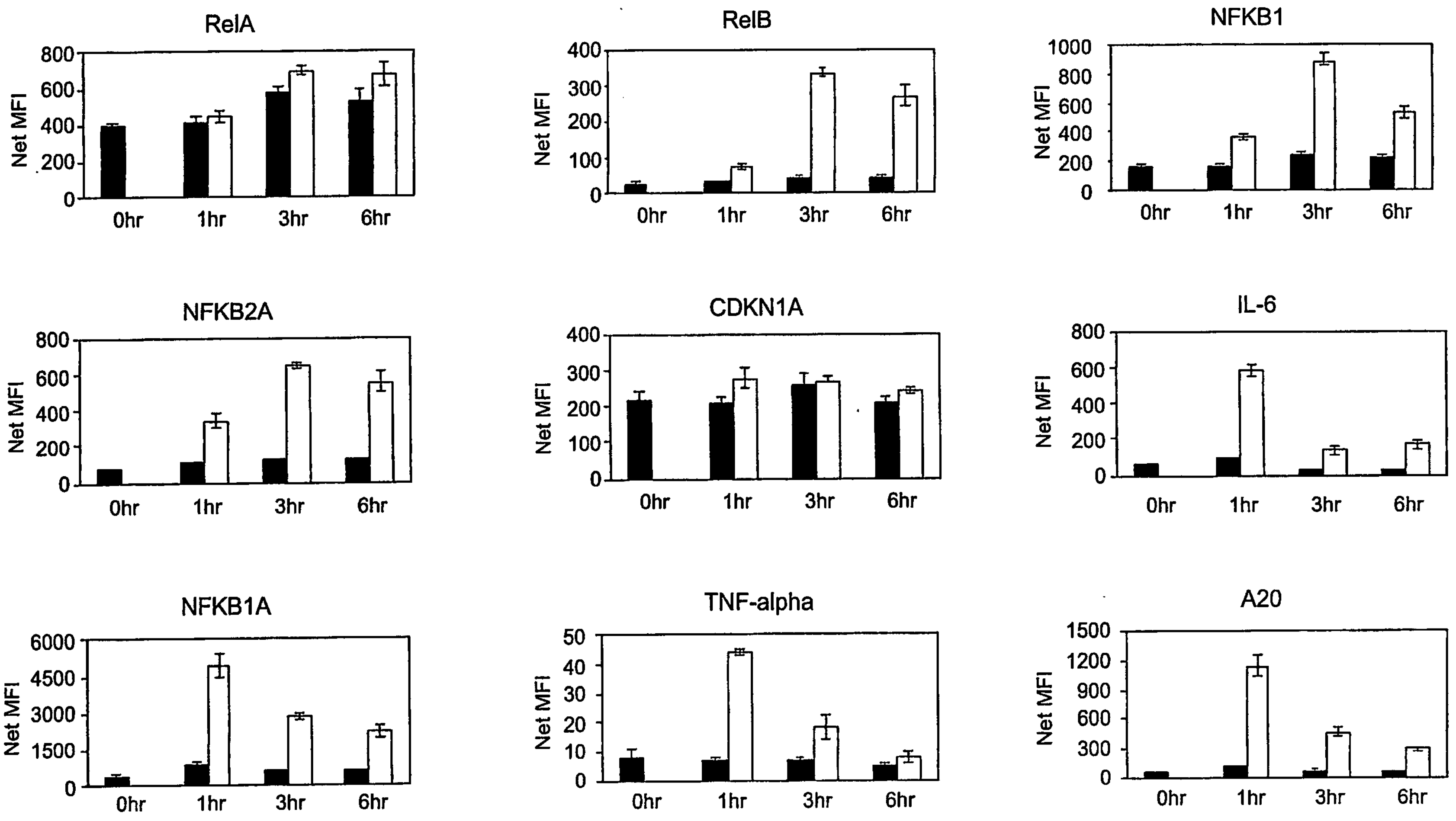
10.0
1.0
IL1B
IL8
GAPD
CSF2
VEGF
IL2
TNF
IL6
IL10
IFNG
☐ Control
■ PMA/LPS

Fig. 8B


RelA
Net MFI
800
600
400
200
0
0hr
1hr
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6hr
RelB
Net MFI
400
300
200
100
0
0hr
1hr
3hr
6hr
NFKB1
Net MFI
1000
800
600
400
200
0
0hr
1hr
3hr
6hr
NFKB2A
Net MFI
800
600
400
200
0
0hr
1hr
3hr
6hr
CDKN1A
Net MFI
400
300
200
100
0
0hr
1hr
3hr
6hr
IL-6
Net MFI
800
600
400
200
0
0hr
1hr
3hr
6hr
NFKB1A
Net MFI
6000
4500
3000
1500
0
0hr
1hr
3hr
6hr
TNF-alpha
Net MFI
50
40
30
20
10
0
0hr
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6hr
A20
Net MFI
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1200
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1hr
3hr
6hr

Fig. 9A

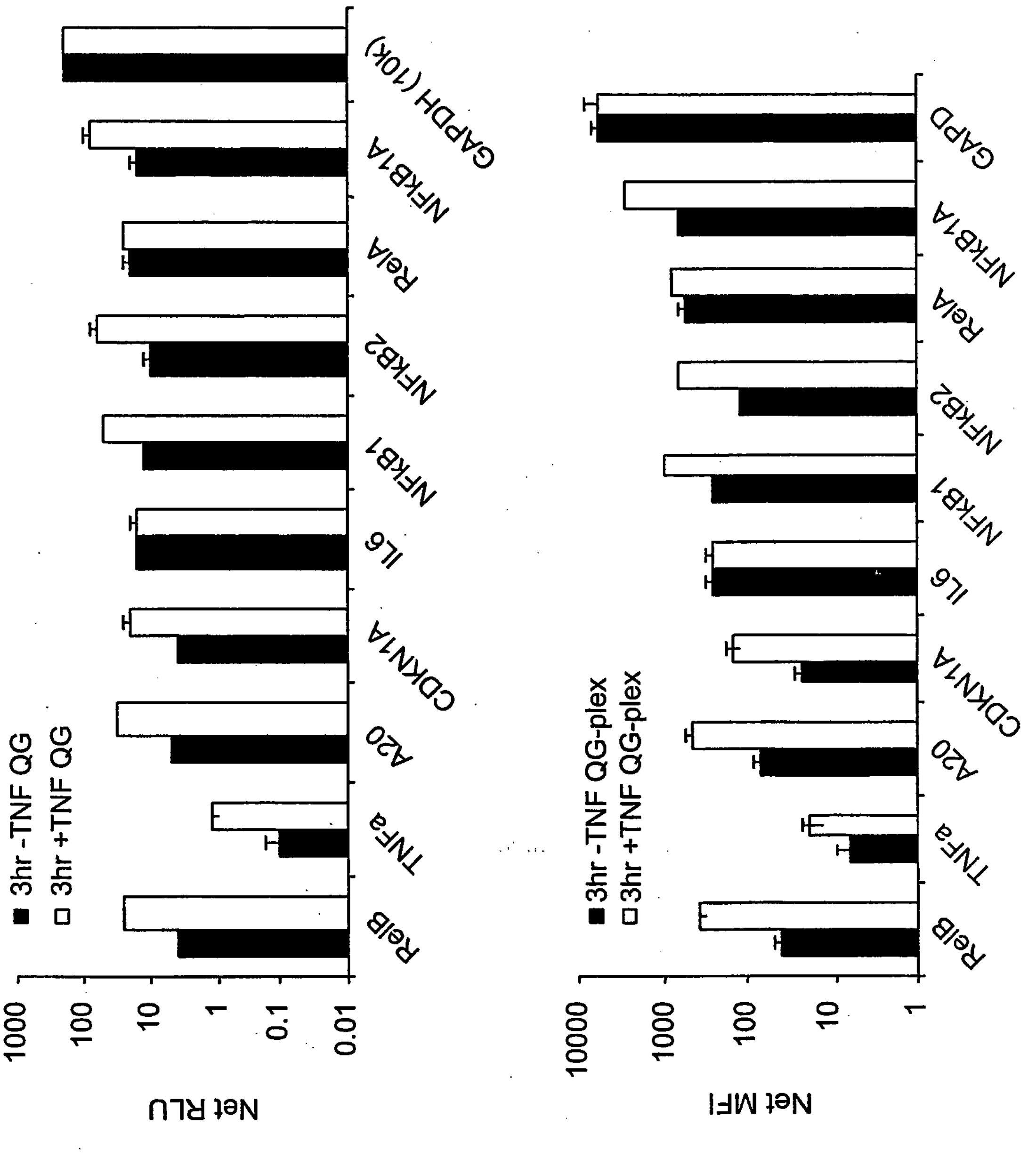
Net RLU
1000
100
10
1
0.1
0.01
■ 3hr -TNF QG
□ 3hr +TNF QG
RelB
TNFa
A20
CDKN1A
IL6
NFKB1
NFKB2
RelA
NFKB1A
GAPDH (10k)
Net MFI
10000
1000
100
10
1
■ 3hr -TNF QG-plex
□ 3hr +TNF QG-plex
RelB
TNFa
A20
CDKN1A
IL6
NFKB1
NFKB2
RelA
NFKB1A
GAPD

Fig. 9B

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